STUDIES ON ANTIBODIES AGAINST MODIFIED DNA FRAGMENTS AND THEIR POSSIBLE ROLE IN THE PATHOGENESIS OF SLE

ABSTRACT

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Abstract

Systemic lupus erythematosus (SLE) is a peculiar autoimmune disorder characterized by the production of anti-native DNA antibodies. The origin of these antibodies and the etiology of SLE has not yet been known. Anti-DNA human autoantibodies show a wide spectrum polyreactivity characterized by their binding with nucleic acids of varying source, size and conformations. The binding of these antibodies to reactive oxygen species (ROS), modified DNA, DNA-psoralen adduct, brominated DNA, RNA-DNA hybrids and various single and double stranded polymers has been reported from various studies. These antibodies also recognize structures other than nucleic acids, e.g. cardiolipin, polylysine and polylysine-polyglutamate complex. Analysis of DNA from SLE patient's plasma has revealed that these molecules are 100-1000 base pair long and rich in G≡C sequences. Such molecules can undergo B-to Z- transition under constrained conditions. SLE is predominantly found in females of child bearing age. Frequent exacerbation of disease during pregnancy or following oral contraceptive therapy strongly suggest the influence of sex hormones on disease activity.

In the present study conformational transition in native calf thymus DNA was induced by bromination under high and low salt conditions and by interaction with polyamines. Attainment of Z-form by native calf thymus DNA was analyzed

by UV and CD spectroscopic techniques. When compared with the spectrum of native DNA, the high salt brominated form exhibited hypochromicity at 260 nm and hyperchromicity at around 295 nm in UV spectral analysis. The changes were less pronounced in the case of native DNA brominated under physiological conditions of saline. These spectral changes indicate that native DNA brominated in high salt has attained left handed Z-conformation. The results were substantiated by the absorbance ratio (A_{295}/A_{260}) of 0.35 in the case of native DNA brominated in high salt, while this ratio was 0.188 for the low salt brominated polymer. Absorbance ratio of 0.3 is a characteristic feature of prototype Z-DNA. Additional evidence for the attainment of Z-form by brominated DNA (4.0 M NaCl) was provided by the inversion of DNA spectrum in CD spectroscopy. This inversion was absent in the case of low salt brominated sample.

Formation of Z-/Z-like DNA as a result of interaction with polyamines was detected by absorbance ratio and binding with anti-Z-DNA antibodies. The specific binding of anti-Z-DNA antibodies to polyamine-DNA complex provided the evidence for conformational isomerization of native B-epitopes into Z-conformation in the presence of increasing concentration of polyamines.

Small size DNA fragments were obtained as a result of controlled digestion with micrococcal nuclease. The

approximate fragment size was determined by standard DNA marker. Native DNA (average size 2oo bp) was separately linked to estradiol-albumin (E_2-BSA) and bovine serum albumin (BSA). Glutaraldehyde was used for covalent coupling. The separation of linked and unlinked species was achieved by gel exclusion chromatography on Sephadex G-200 column. The BSA-DNA and E_2-BSA-DNA conjugates were characterized by ultraviolet spectroscopy wherein a red shift of 8 nm and 11 nm in the minima was respectively observed for DNA-BSA and E_2-BSA-DNA conjugates.

Structural change or perturbations in native DNA and native DNA (~200 bp) as a result of modification were also checked by mid-point melting temperature (Tm) analysis and evaluation of various thermodynamic parameters. As revealed by the values for Gibb's free energy (ΔG_D), all the modified forms, with the exception of DNA-BSA conjugate, were thermodynamically stable than their corresponding DNA controls.

Antibodies against E_2-BSA, E_2-BSA-DNA and DNA-BSA conjugates were raised in rabbits. Direct binding ELISA showed a titer> 1:12800 for anti-E_2-BSA and anti-E_2-BSA-DNA antibodies. The DNA-BSA conjugate induced antibodies with a titer greater than 1:1600. The specificity of induced antibodies was confirmed in inhibition ELISA by using the respective immunogens as inhibitors in each case. The

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In conclusion, native DNA and small size DNA fragments have interspersed base pair sequences which have the potential to undergo B-to Z- or Z-like transition. These conformationally altered segments could be acting as autoantigens for the etiopathogenesis of SLE. The results also draw attention towards the possible role of female sex hormone in the induction of anti-DNA response, the characteristic feature of SLE.


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CERTIFICATE

I certify that the work presented in the following pages has been carried out by **Moinuddin** and is suitable for the award of Ph.D. degree in Biochemistry of the Aligarh Muslim University, Aligarh.

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Dedicated

to my

Parents and Teachers

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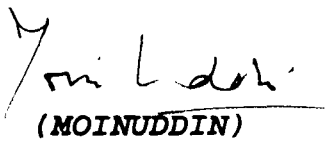

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CONTENTS

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ABBREVIATIONS

A_{260}	=	Absorbance at 260 nm
A_{280}	=	Absorbance at 280 nm
A_{295}	=	Absorbance at 295 nm
Br-DNA	=	Brominated DNA
Br-4	=	Native DNA brominated in 4.0 M NaCl
Br-N	=	Native DNA brominated in 0.15 M NaCl
bp	=	Base pair
CD	=	Circular dichroism
DEAE	=	Diethylaminoethyl
ds DNA	=	Double stranded DNA
EDTA	=	Ethylenediaminetetra-acetic acid
E_2-BSA	=	Estradiol-albumin
IgG	=	Immunoglobulin G
4MU-P	=	4-methyl umbelliferyl phosphate
PAGE	=	Polyacrylamide gel electrophoresis
PNP-P	=	*p*-nitrophenyl phosphate
SLE	=	Systemic lupus erythematosus
ssDNA	=	Single stranded DNA
UV	=	Ultraviolet
ug	=	Microgram
ul	=	Microlitre

LIST OF FIGURES

LIST OF TABLES

Introduction

Ever since it was first isolated from pus cell nuclei (Miescher, 1879), the DNA has occupied a centre stage in various biochemical investigations. In fact the modern era of molecular biology began with the elucidation of double helical structure of DNA (Watson and Crick, 1953) by the detailed analysis of diffraction pattern and by careful molecular modelling. While this model of DNA, also known as B-DNA is regarded as the most accepted structure of DNA molecule in solution, more sophisticated x-ray diffraction studies have thrown light on various alternative conformations of DNA such as A- and C-DNA within the right handed family (Adams et al., 1981) (Table 1). This conformational flexibility has been the most important feature of the double helix. It is a dynamic structure which can adopt multiple conformations depending on its bioenvironment. These alternate conformations are likely to play a crucial role in gene expression by increased or decreased synthesis of regulatory proteins. Over the last few years important progress has been made in understanding the nature of DNA fine structure. Since the first direct demonstration of sequence dependent heterogeneity by single crystal x-ray analysis (Dickerson and Drew, 1981), many experimental techniques have demonstrated the effects of base sequence on the properties of DNA. Certain base sequences can give rise to very particular conformations and

TABLE 1

Different Conformations of Right-Handed DNA

Form	Pitch (A°)	Residue per turn	Inclination of base pair from horizontal
A (Na-salt, 75% relative humidity)	28	11	20°
B (Na-salt, 92% relative humidity)	34	10	0°
C (Li-salt, 66% relative humidity)	31	9.3	6°
RNA-DNA hybrid	28	11	20°

Source : Adams et al. (1981)

may also influence the local flexibility of the DNA double helix. DNA is no longer considered a static molecule, but rather, a dynamic structure in which different conformations are in equilibrium with each other (Rich et al., 1984; Pecheneya, 1993). It has now become clear that conformational diversity of the DNA helix may play an important role in the recognition of DNA binding sites by proteins and other important biomolecules (Synder et al., 1986; Zahn and Blatter, 1987; Stenzel et al., 1987). The static image of DNA-protein interaction has been replaced by a more global and dynamic view in which proteins and other molecules recognize the overall 3-dimensional form of DNA, its local flexibility and both the interacting partners can undergo considerable structural modification at the moment of complex formation (Drew and Travers, 1984; Majumdar and Adhya, 1989). Antibodies generated against various modified conformations of DNA can act as a tool to probe the presence of these forms in the body.

Antigenicity of DNA

Antibodies to DNA (anti-DNA) were first reported in the sera of patients with systemic lupus erythematosus in 1957. Since then the immunogenecity of nucleic acids has received considerable attention. Native DNA in B-conformation is non-immunogenic despite the fact that most nucleic acids elicit the formation of antibodies (Stollar, 1986). Even MRL mice,

which are genetically programmed to "spontaneously" produce anti-B-DNA antibodies do not respond to exogenous DNA and fail to elicit high levels of antibody against double stranded DNA (Hughes, 1986). Animals immunized with DNA show only limited antibody production, almost exclusively directed against single stranded DNA (Plescia et al., 1964). A decisive breakthrough in the production of anti-DNA antibodies was reported in 1960 by Levine et al. They successfully elicited antibodies directed to the denatured (single stranded) DNA of T- even bacteriophage. The antibodies were directed to the modified base of these phages as only they seemed essential for the production of antibodies, because immunization with purified DNA having normal bases yielded little or no antibody. Two general methods for the generation of anti-nucleic acid antibodies have been experimentally demonstrated (Plescia et. al., 1964). One is the use of bases, nucleosides and nucleotides as haptens (Erlanger and Beiser, 1964). The resulting antibodies react with denatured DNA with considerable selectivity for the corresponding purine and pyrimidine bases. The second general method is the formation of immunogenic complexes of nucleic acid and a positively charged protein carrier (Plescia et al., 1964). This has provided an immunogenic stimulus for denatured DNA, synthetic homopolynucleotides, chemically modified DNA and

certain helical synthetic polynucleotides. Effective helical immunogens in this form have been double stranded RNA, RNA-DNA hybrids, left handed Z-DNA, triple helical RNA and DNA analogues and double helical polydeoxyribonucleotides that differ from B-DNA, such as poly(dG).poly(dC) (Stollar, 1973; 1975; 1986; Anderson, 1988). An important property of the resulting antibodies is that they do not recognize B-conformation of DNA. Immunization with nucleic acid-MBSA (methylated BSA) complexes generally has not been effective for native DNA, tRNA or ribosomal RNA (Stollar, 1989). Antibodies of high selectivity have been induced by left handed Z-DNA and these antibodies have served as a tool for the identification of Z-DNA in chromosomes (Lafer et al 1981, Stollar, 1986). Injection of histone-DNA complexes yielded some anti-histone but not anti-DNA antibodies (Stollar and Ward, 1970), nor did immunization with isolated nucleosomes induce anti-DNA response (Einck et al., 1982). A duplex DNA, poly(dT-dC).poly (dG-dA) was used by Lee et al. (1985) to generate monoclonals. The antibodies so obtained gave a weak cross reaction with native calf thymus DNA. A monoclonal IgM antibody selective for native DNA was obtained from a mouse immunized with DNA and several different synthetic DNA analogues (Huang et al., 1985). Recently it was observed that, injection of bacterial native DNA into normal mice induced antisera that bound native

bacterial but not the mammalian DNA (Gilkeson et al., 1989). Injection of BK virus into rabbits induced antibody to both native DNA and histone (Flaegstad et al., 1988). A much smaller population of antibodies against native DNA was obtained by immuno- purifying the antibodies generated against deoxyribonucleotide-protein conjugate, prepared with the help of water soluble carbodiimides (Jacob and Jacob, 1985).

Antibodies have also been generated against chemically modified and triple helical DNA. Immunization with methylated BSA complexes of modified DNA, as with cisplatin-modified DNA (Poirier, 1981; Sundquist et al., 1987) or with modified nucleosides or nucleotides conjugated to proteins has led to the induction of antibodies (Muller and Rajewsky, 1980). A triple helical DNA can be formed at neutral pH by a mixture of poly (dT-dm^{5}C) and poly (dG-dA) (Lee et al., 1980). Monoclonal antibodies generated against this complex polymer, recognized the antigen but failed to react with other related polynucleotides that cannot form a triple helix (Lee et al., 1987). As with other nucleic acid configurations, the antibodies generated either against chemically modified DNA or against triple helical DNA do not react with native B- helical DNA.

Recently it has been found that sera from normal subjects showed binding to highly purified double-stranded

(ds) DNA from *Micrococcus lysodeikticus* (Bunyard and Pisetsky, 1994). The results indicate that sera from normal human subjects have antibodies which bind bacterial ds DNA and extend the range of determinants that can be recognized as foreign by the normal immune system. To explain this response, it has been postulated that bacterial DNA can induce antibody production during infection because of sequences rarely present in host DNA and therefore recognized as foreign (Pisetsky et al., 1990).

Conformational Substates in B-DNA

Native DNA in B-form is a poor immunogen. Certain right handed helical double stranded polydeoxy-ribonucleotides differ slightly from the average B-DNA helix and are immunogenic beyond the adjuvant induced autoantibody expansion (Madaio et al., 1984). The immunogenicity is itself an indication of their having a structure other than the B-form. They elicit antibodies that recognize the differences between them and native B-DNA. The antibodies have been found to react with the immunogen, but their binding to native DNA is either very weak or not at all. This again points towards the existence of structural differences between the helices. These immunogenic analogues of natural B-form of DNA include, poly(dG). poly(dC) (Stollar, 1970); poly(dG-dA). poly(dT-dC) (Lee et al.,

1985); poly.(dA). poly (dT) (Arnott et al. 1983; Diekmann and Zarling, 1987); poly(dG-dC) (Zarling at al., 1987). The other right handed structures within naturally occurring DNA which arise as a result of change in humidity and salt conditions are A- and C-DNA (Arnott and Hukin, 1972; Arnott and Selsing, 1975; Saengar, 1984). Under conditions, such as addition of ethanol or trifluoroethanol, which decreases water activity, the B→A transition can be induced in solution (Ivanov and Krylov, 1992). The A- configuration is also found in double stranded RNA and RNA-DNA hybrid (Spencer et al., 1962; Milman et al., 1967). There is strong evidence that the conformation of poly(dG). poly (dC) in solution is A-like (Arnott and Selsing, 1974; Sarma et al., 1986). The proposal of Stafford and Donnellan (1968), that DNA in dormant spores of Bacillus species is entirely in an A-like conformation, has still been found valid and has been reviewed in the light of recent evidences (Setlow, 1992). Relatively short stretches of the sequence $(dC\text{-}dA)_n.(dG\text{-}dT)_n$, which is the most abundant purine-pyrimidine dinucleotide repeat in eukaryotic genome, have been found in all eukaryotes, from yeast to human beings (Hamada et al., 1982; Weber 1990). Studies on potential Z-forming sequence $(dT\text{-}dG)_n.(dC\text{-}dA)_n$ has revealed evidence for an "alternating B" conformation on the nucleosomal surface (Gross et al., 1985). It is possible that the alternative helical structure

of these sequences yields a distinctive structure in the nucleus that is recognized by proteins functioning in recombination and replication events. Because these sequences exhibit length polymorphism, they represent a new rich source of informative genetic markers (Weber and May, 1989). An "alternating B" structure has been proposed for poly(dA-dT). poly(dA-dT) in solution. This structure is based on DNase I cleavage pattern, proton and ^{31}P nuclear magnetic resonance (NMR) spectroscopy, and X-ray fiber diffraction (Klug et al., 1979). The synthetic deoxyoctanucleotide (G-G-G-G-T-C-C-C) crystallizes as an A-type DNA double helix containing two adjacent G-T base pair mismatches (Kneale et al., 1985). Also, the potentially Z-DNA forming sequence d(GTGTACAC) crystallizes as A-DNA (Jain et al., 1987). Poly (dG). poly(dC) sequences, under torsional stress, induce an altered DNA conformation upon neighbouring DNA sequences and such altered DNA structures may play an important role in chromatin function (Shigematsu and Kohwi, 1985). Studies on DNA-drug interaction have shown that because of drug binding, a fraction of DNA is locked in the B-form and is incapable of adopting the A-form even under conditions of rigorous dehydration (Fritzsche, 1991; 1994).

Left Handed Double Helical DNA

DNA is a very dynamic molecule which can adopt both right handed and left handed conformations. This is of considerable interest as biological function at molecular level is often closely related with the structure. A number of variations of the basic structure of DNA, i.e. the B-form, have been described in which there is some alteration in the form of the molecule. An alternate purine-pyrimidine copolymer, poly(dG-dC). poly(dG-dC) has been shown to undergo salt induced conformational transition in neutral solution (Pohl and Jovin, 1972) and in 50% ethanol (Pohl, 1976). The left handed form has also been described in a single crystal of a DNA fragment containing six base pairs with the sequence d(CpGpCpGpCpG) (Wang et al.,1979). The crystal contained magnesium and spermine as cations. The ribose phosphate backbone in the left handed double helical form pursues a zigzag course, leading to the name, Z-DNA. It is a prototype left handed double helix whose structural properties differ significantly from those of the classical B-form.

Studies carried out for the formation of Z-DNA *in vitro* have been successful in inducing conformational transition in various right handed polynucleotides. The linear duplex (dG-dC)n can undergo B→Z transition at NaCl concentration above 2.25M (Klysik et al., 1981; Pohl, 1983) or as a result

of modification of either guanine or cytosine residues (Sage and Leng, 1980; Behe and Felsenfeld, 1981; Klysik et al., 1983). Other DNA sequences with alternating purine-pyrimidine residues, including poly(dA-S^4dT) and poly(dT-dG).poly(dC-dA), exhibit fiber structures that are compatible with left handed Z-form as well (Arnott et al., 1980). Poly(dG-m^5dC) converts readily to left handed Z-DNA *in vitro* in the presence of millimolar concentrations of divalent metals and polyamines, as well as small peptides (Behe and Felsenfeld, 1981; Rich et al., 1984; Takeuchi et al., 1991). The above sequences and certain others that contain alternating purine-pyrimidine sequences can adopt the Z-conformation in response to negative supercoiling or elevated ionic strength (Peck et al., 1982; Gross et al., 1985).

It has been shown that different lysine rich histones can cause varying conformational changes in the condensation of chromatin in DNA regions of highly biased base sequence (Mura and Stollar, 1984). In addition, naturally occurring polyamines, putrescine, spermidine and spermine can induce B to Z transition of poly(dG-m^5dC).poly(dG-m^5dC) at micromolar concentrations (Behe and Felsenfeld, 1981; Thomas et al., 1985). B to Z transition has also been reported in poly (dA-dC). poly (dG-dT) at low ionic concentrations in the presence of spermidine and spermine (Thomas and Messner,

1986). While most of the conditions used to induce Z-DNA formation in the laboratory are not physiologic, polyamines can induce and stabilize Z-DNA form of conformationally labile polynucleotides under physiologically relevant cation concentrations (Thomas et al., 1985; Thomas and Messner, 1988). Hasan and Ali (1990) reported the presence of Z-like features in calf thymus DNA that was brominated under high salt (4M NaCl) conditions.

Various studies performed on conformationally different polynucleotides under elevated ionic conditions have successfully described the attainment of Z-conformation. The question that now strikes our mind is whether Z-DNA can occur *in vivo* or not. A number of studies suggest that Z-DNA may exist *in vivo* (Jaworski et al., 1987; Rahmouni and Wells, 1989; Wittig et al., 1989; Rahmouni, 1992), however, the extent of its occurrence is yet to be determined. Inside the cell, Z-conformation can be induced as a result of negative supercoiling which is generated during transcription (Liu and Wang, 1987; Tsao et al., 1989). Furthermore, the equilibrium between B and Z-DNA can be influenced by proteins that preferentially bind to one of the two conformations (Lafer et al., 1985). Z-DNA has been implicated in some important biological processes, such as general DNA recombination (Bullock et al., 1986; Treco and Arnheim, 1986; Blaho and Wells, 1987; Wahls et al., 1990),

and both positive and negative transcriptional regulation (Nordheim and Rich, 1983; Naylor and Clark, 1990). There are many approaches that can be adopted to further understand the biological significance of Z-DNA *in vivo*. One approach is purification and *in vitro* characterization of a protein that has got enhanced affinity for left handed DNA. Zhang et al. (1992) have purified and characterised a putative Z-DNA binding protein, Zuotin, in *Saccharomyces cerevisiae*. Recently, it has been reported that upon a linear increase of the ionic strength, the hexadecamer $d(C-G)_8$ undergoes the expected B to Z transition, which is completed at 2.0 M $MgCl_2$. But as the salt concentration is raised above 2.0 M, a second structural transition from Z-DNA back to right handed B-DNA is observed. This Z to B transition can be induced by divalent ions (e.g. $CaCl_2$, $MgCl_2$) but not by monovalent NaCl (Reich et al., 1993).

Immunochemical studies have revealed that Z-DNA is a powerful immunogen eliciting a highly specific antibody response (Lafer et al, 1983a; Zarling et al., 1984). Both polyclonal and monoclonal antibodies have been induced with several forms of Z-DNA (Stollar, 1986). Some of the antibodies show selectivity for a particular base sequence, whereas others are more general anti Z-DNA antibodies (Lafer et al., 1983a; Nordheim et al., 1986). Polyclonal sera induced by brominated poly(dG-dC) fall in the later

category, while monoclonal antibodies from mice immunized with brominated poly(dG-dC), however, may have either kind of reactivity. One monoclonal antibody, Z-22 reacts with Z-DNA of variable sequence, whereas another, Z-44, is selective for Z-DNA formed by (dG-dC) sequences, it does not react, for example, with poly (dG-dm^{5}C) or poly (dT-dG). poly(dC-dA) or other mixed base sequences in the Z-helical form (Nordheim et al., 1986). Antibodies against Z-DNA have served as probes in the search for naturally occurring left handed helix. Antibodies have identified Z-DNA sites in supercoiled plasmid and viral DNAs (Miller et al., 1983; Nordheim and Rich, 1983; Hagen et al., 1985) and have served to titrate the transition from the B-form to Z-form as a function of superhelical density (Nordheim et al., 1982) or counterion or polyamine concentration (Thomas et al, 1988). They have also been used to identify and isolate Z-DNA regions from a library of E. coli genomic DNA (Thomae et al, 1983; Hoheisel and Pohl, 1987). A left handed Z-RNA has also been identified with poly (rG-rC) and corresponding antibodies have identified this structure in cytoplasm by immunofluorescence (Zarling et al., 1987).

Possible Biological Role of Z-DNA

The functional significance of the Z-DNA remains a provocative and controversial issue. Several observations, however, hint towards the possibility that left handed

motifs might be involved in the regulation of biological processes either directly by acting as potential templates for RNA polymerase (Sande and Jovin, 1982; Hipskind and Clarkson, 1983; Nordheim and Rich, 1983) and by modulating strand exchange (Fishel et al., 1988) thus affecting transcription and recombination processes or indirectly by altering the extent of DNA packaging (Castleman et al., 1984; Thomas and Bloomfeld, 1985; Reich et al, 1991) and supercoiling (Peck et al., 1982; Rich et al., 1984). The question that naturally arises is what physiological processes act *in vivo* to generate negative supercoiling that is continuously being removed by the activity of topoisomerase I (Zhang et al., 1988). Wittig et al.(1991) have suggested that transcription may be one of the physiological processes that generate negative supercoiling and this stabilizes Z-DNA formation in the intact cell. Conformational diversity of the DNA helix appears to be used quite extensively by living organisms with regard to protein- DNA recognition and hence function (Traverse, 1989). In several cases, DNA recognition by proteins seems to rely heavily, if not entirely, on DNA conformations and flexibility as opposed to base sequence *per se* (Otwinowski et al., 1988; Goodman and Nash 1989).

Polyamine-Nucleic Acid Interactions

Polyamines are ubiquitous polycationic metabolites in prokaryotic and eukaryotic cells. Their roles in the regulation of major functions in cell growth, cell differentiation and their intricate and exquisitely regulated biosynthetic pathways have attracted considerable attention during the last few decades. Various books and reviews have dealt in length with these aspects, including the design of enzyme inhibitors which by depleting cells of their normal polyamine contents provoke cell growth arrest and other pharmacologically important phenomena (Heby, 1981; Pegg and Mc Cann, 1982; Tabor and Tabor, 1984; Pegg, 1986, 1988; Mc Cann et al., 1987). Polyamines are found to be associated with ribosomes and membranes. Their precise function inside the cell is not fully understood. They are reported to have an antimutagenic effect and prevent dissociation of 70S ribosomes to 50S and 30S subunits. They increase the resistance of protoplasts to osmotic lysis. Polyamines stimulate translation (initiation and elongation) of the peptide chain and are implicated in maintaining translational fidelity, ribosome structure and stability (Marton and Morris, 1987) thus markedly influencing protein synthesis in both prokaryotes and eukaryotes. In various growing tissues, polyamine synthesis accompanies or precedes nucleic acid synthesis (Mc Cann et al., 1972, Heby et al.,

1976). The increased synthesis of RNA also depends on the stimulating effect of polyamines on RNA polymerase. The concentration of polyamines is elevated in biological fluids and tissues in a number of disease states such as cancer, sickle cell anemia, cystic fibrosis, psoriasis and systemic lupus erythematosus (Puri et al., 1978; Gunnia et al., 1991). Similarly, the polyamine levels in the spleen cells of MRL-lpr/lpr mice are about three times higher than that in the spleen cells of normal mice (Bowlins et. al., 1986; Thomas and Messner, 1989).

Spermine and spermidine are known to be widely distributed in nature. Spermine is the most abundant polyamine found in animal cells (Tabor and Tabor, 1984). Spermidine or spermine is essential for aerobic growth of *Saccharomyces cerevisiae* (Balasundaram et al., 1991). The amount of spermidine varies inversely with the amount of Mg^{2+} in ribosomes, and 30S and 50S ribosomal subunits remain associated in the absence of Mg^{2+} if spermine is present.

Although the functions attributed to the polyamines are numerous, their modes of action at molecular level remain a matter of speculation. Because of their protonated amino groups (at physiological pH values), polyamines are known to bind to negatively charged cellular macromolecules such as nucleic acids. The interaction of polyamines with nucleic

acids may be responsible in part for the biological functions of polyamines (Basu et al., 1989). Association of polyamines with specific base sequences of nucleic acids has been found in crystals (Prinz et al., 1976; Quigley, 1982) and in solution (Igarashi et al., 1977; Marton and Feuerstein, 1986; Basu et al, 1988). Thermophilic bacteria growing under extremes of heat are known to produce a variety of polyamines to protect their genetic material from denaturation (Oshima, 1982). The polyamines composition of the extreme thermophile depends on growth temperature (Oshima and Baba, 1981). The thermophilic cells grown at an optimum temperature (75°C) contain two novel tetramines, thermine (Oshima, 1975) and thermospermine (Oshima, 1979). Bordin et al. (1992) have suggested that different DNA superstructures are relevant in interactions with polycations, such as spermine. It has also been reported that spermine binds in the deep groove of the A-DNA octamer d(GTGTACAC) by interacting with the bases (Jain et al., 1989). Recent evidences indicate that polyamines play an important role in the growth of hormone responsive breast cancer (Kendra and Katzenellenbogen, 1987; Manni et al., 1992). The interaction of polyamines with DNA produces profound changes in the structural organization and reactivity of DNA. These include, the stabilization of double stranded DNA, collapse of DNA into toroids and

spheroids (Gosule and Schellman, 1978; Thomas and Bloomfield, 1983) and the induction of left handed conformation in alternating purine-pyrimidine sequences and plasmids containing blocks of these sequences (Behe and Felsenfeld, 1981; Thomas and Messner, 1988; Thomas et al., 1991). Polyamines have been shown to promote DNA aggregation, to stabilize DNA against thermal denaturation, enzymatic degradation, and shear-induced strain, to induce the *in vitro* condensation of DNA, and to facilitate the transition of DNA from B-toZ-conformation (Vertino et al, 1987; Feuerstein and Marton, 1987). It has also been suggested that polyamines may stabilize certain polynucleotides, e.g. poly(dA-dT).poly(dA-dT) or poly(dG-dC).poly(dG-dC), in a conformation which has reduced affinity for ethidium bromide (Delcros et al., 1993). A conformational transition is induced in poly (amino^{2}dA-dT).poly(amino^{2}dA-dT) by micromolar concentrations of polyamines (30 μM) in low salt aqueous solution. The resulting conformer is suggested to be an A form (Garriga et al., 1993).

Naturally occurring polyamines have been reported to induce B to Z transition of poly(dG-m^{5}dC).poly(dG-m^{5}dC) at micromolar concentrations (Behe and Felsenfeld, 1981; Thomas et al, 1985). Polyamine induced Z-DNA formation provides a potential pathway for Z-DNA induction and stabilization

under physiologically relevant conditions. Thomas and Messner (1986) have shown that poly (dA-dC). poly(dG-dT) undergoes B to Z transition at low ionic concentrations in the presence of spermidine and spermine. This finding assumes significance because in contrast to the rare occurrence of blocks of $(dG\text{-}dC)_n.(dG\text{-}dC)_n$ segments, blocks of $(dA\text{-}dC)_n.(dG\text{-}dT)_n$ are widely dispersed in native DNAs (Hamada and Kakunaga, 1982; Hamada et al., 1982).

Highly sensitive enzyme immunoassay techniques have demonstrated (Thomas et al., 1988) that, in the absence of polyamines, a polynucleotide sequence, poly(dG-m^5dC).poly(dG-m^5dC) does not bind to monoclonal anti-Z-DNA antibody (Z 22), but rapid interactions occur when polyamines are added to the system with gradual increase in concentration (Thomas and Messner, 1988).

Klevan and Schumaker (1982) observed that addition of poly-L-arginine but not poly-L-lysine to poly(dG-dC).poly(dG-dC) induces Z-conformation which is retained even after dialysis to low salt. This observation suggests that Z-DNA stabilization does not solely depend on simple electrostatic factors but may also involve some specificity in the interaction of arginine side chains with Z-helix. Analysis of interactions of H1 and H5 histone with polynucleotides of B-and Z-conformations revealed two things. Firstly, at low protein:DNA ratios, both H1 and H5

bound more Z-DNA than B-DNA. Secondly, the binding of Z-DNA was less sensitive to interference by an increase in ionic strength (to 600 mM NaCl). Moreover, H5 histone caused more profound C.D. spectral changes than did H1 (Mura and Stollar, 1984). These findings demonstrate the ability of different lysine rich histones to cause varying conformational changes in the condensation of chromatin in DNA regions of highly biased base sequences. The occurrence of H5 is characteristic of cells which are inactive in both transcription and replication (Billet and Hindley, 1972; Urban et al., 1980).

Recent studies have suggested that natural polyamines are capable of altering the immunogenicity of polynucleotides by mechanisms involving the stabilization of Z-DNA conformation (Gunnia et al., 1991). These results may have direct implications in the recent findings of high levels of polyamines and anti-Z-DNA antibodies in the sera of lupus patients and autoimmune mice, suggesting the involvement of polyamines in altering the topological state of DNA (Sehgal and Ali, 1990; Thomas et al., 1990).

Autoimmunity and Autoimmune Diseases

The presence of antibodies in the sera of physiologically healthy individuals can be traced back to as early as 1900 when Landesteiner and his group reported the interaction of such antibodies with blood group determinants

of A-B-O system. Subsequent studies have demonstrated the presence, in the sera of normal humans as well as that of several other animal species, of antibodies reacting with a variety of cellular and humoral constituents. These antibodies, subsequently termed natural antibodies were found, in some case, to react with self constituents, such as sperm, brain, and skin antigens, that under normal physiological conditions are not found in the circulation and therefore not exposed to the immune system. Natural antibodies are roughly of two types, one with a well defined and high specificity and another with a weak specificity. Boyden (1963) suggested that natural antibodies with well defined specificity may correspond to induced antibodies appearing after an antigenic stimulation, whereas natural antibodies with weak specificities may correspond to spontaneously appearing antibodies, i.e. those without the need for any known antigenic stimulus. In the 1960s, with the general acceptance of Burnet's clonal selection hypothesis, the studies on spontaneously occurring antibodies diminished considerably. This theory put forth the idea that there are lymphocytes which proliferate into antibody producing cell when they come in contact with a 'non-self' antigen. Lymphocytes capable of reacting with self components are deleted during embryonic life and provide self tolerance. On that basis, synthesis of

autoantibodies reacting with self components does not correspond to a physiological process but is the result of only pathological situations (Burnet, 1959).

The function of the immune system is to discriminate between self and non-self. Normally, the immune system responds to a wide variety of foreign insults, such as bacteria, viruses, parasites, and internal changes such as cancer, while not responding to one's own self antigens. However, under varied stresses, the immune system fails to discriminate between 'self' and 'alien', leading to antibody production against various cellular components. The term autoimmune disease, refers to a variety of illnesses in which immune processes are directed against the self. They may be predominantly humoral (antibody) or cellular in origin, or a combination of both. A hallmark of most autoimmune diseases is the presence of autoantibodies against self antigens. An autoimmune disease may be organ/tissue specific e.g. diabetes, autoimmune thyroiditis, Goodpasture's disease or primary biliary cirrhosis or non-organ/tissue specific (systemic) such as progressive systemic sclerosis (PSS) or systemic lupus erythematosus (SLE) in scope. It may destroy, mimic, or enhance the target. It can range in severity, from being only an irritant to being fatal to the individual (Minard, 1989).

The potential for autoimmunity is present in normal

individuals and each can become autoimmune under abnormal circumstances. Autoimmunity is prevented by proper immune regulation which is mediated by various T-lymphocyte sub-populations. Helper and suppresser T-cells act together to establish an immunoregulatory balance, which determines the level of immune response to a particular antigen. A disequilibrium resulting either in the generation of helper T-cells, or in a deficiency of suppressor T-cells, could trigger potentially autoreactive B-cell clones to autoantibody production. Primary variations of the target organ can either be genetically determined changes of autoantigenic determinants or secondary alterations based on chemical, viral or other environmental factors.

Possible Causes of Autoimmune Diseases

Despite enormous knowledge accumulation about immune reactions, the etiology of autoimmunity is still an enigma. Based on various theories, the possible factors are discussed below.

Immunoregulation - The malregulation of the immune system depends on multiple components of the feedback regulation system including T cells, B cells, and idiotopes (Shoenfeld and Schwartz, 1984). There are many components which have not been identified yet that can up or down-regulate cells of the immune system. Normally B-cells with potential to

produce autoantibodies are held in-check by a lack of helper cells or an abundance of suppressor cells. In autoimmunity, an imbalance of this regulatory system becomes manifest. Abnormalities of immune regulation in PSS due to an excess of helper cell function have been demonstrated (Krakauer et al, 1981), and diseases with depressed suppressor cell function, including SLE, diabetes mellitus, Graves'disease, multiple sclerosis (MS), myasthenia gravis (MG), RA, PSS, and primary biliary cirrhosis have been described (Shoenfeld and Schwartz, 1984). In normal individuals, the T-helper to T-suppressor ratio is 2 : 1 in the peripheral blood, whereas in autoimmune diseases, this ratio shoots to 15:1 (Gray et al., 1987; Morimoto et al., 1987; Shivkumar et al., 1989). Because of complexity of the regulatory system, many factors can contribute towards an imbalance. Such an imbalance could be determined in part by the genetic make up and HLA types of the individual. The possibility of sporadic mutations in some individuals cannot be ruled out, though the mechanisms by which these mutations occur are not well understood. Various foreign organisms may upset the balance of immunoregulation. For example, HIV infection causes a switch from a T_H1 like lymphokine pattern to a T_H2 type pattern. This switch is paralleled by an increased number of B cells and hypergammaglobulinemia, which is commonly seen in autoimmune diseases as well (Levinson, 1994).

Tolerance- Tolerance is a state of immunologic nonresponsiveness to substances that would be expected to evoke an immune response, like one's own antigens. The impairment of self tolerance allows for autoimmune disease. Tolerance is accomplished through three mechanisms: thymic education, thymic deletion, and peripheral tolerance. Thymus is the major site for self-nonself discrimination. It is here that the T-cell repertoire is determined. Some cells are programmed to die (negative selection), while others are not (positive selection) (Adorini, 1993). Those with a high affinity for self ligands are negatively selected. It has also been suggested (Rose, 1994) that thymus is the critical time keeper with ageing process with respect to immune responses. Because the thymus involutes asymmetrically, a clonal imbalance occurs with ageing, since the proportion of autoantigen-specific helper/inducer T cells increases as a function of autoantigen specific regulatory T cells. Thus, circulating autoantibody level rises with age. As the thymic cortex atrophies, the response to foreign antigens declines, whereas the response to self antigens rises, generating the ageing paradox.

Viral factors- Viruses have close association with autoimmune diseases of humans and animals (Steinberg et al., 1990; Kreig and Steinberg, 1990; Kreig et al., 1989; 1991). Viral infections can promote autoimmune reactions by varied

mechanisms. It is conceivable that infectious antigens could cause an immune response aimed towards destroying the infectious agent that could lead to cellular damage and autoimmune disease. Molecular machinery can be another factor responsible for autoimmune reactions as a result of viral infection. In such a case, there is similarity between stretches of amino acid or other sequences in molecules of the infectious organisms and molecules of the host, e.g. adenovirus type 2 has sequences very much like those of myelin basic protein (Steinman, 1993). In responding to the virus, the immune system may generate antibody response against the self molecule.

Epstein-Barr virus (EBV) has been most prominently considered as a cause of autoimmune disease because of its ubiquity, persistence and ability to act on the immune system. For example, EBV acts as a polyclonal B cell activator, stimulating mitosis and immunoglobulin secretion as well as promoting autoantibody production, especially rheumatoid factor (Gregory et al., 1991).

Genetic factors- Autoimmune diseases, in general, show a highly significant familial predisposition. Relatives of a patient suffering from autoimmune disease are at high risk of developing similar autoantibodies as compared to general population (Arnett and Shulman, 1976; Hochberg, 1987; Arnett, 1992). The majority of the genes which have been

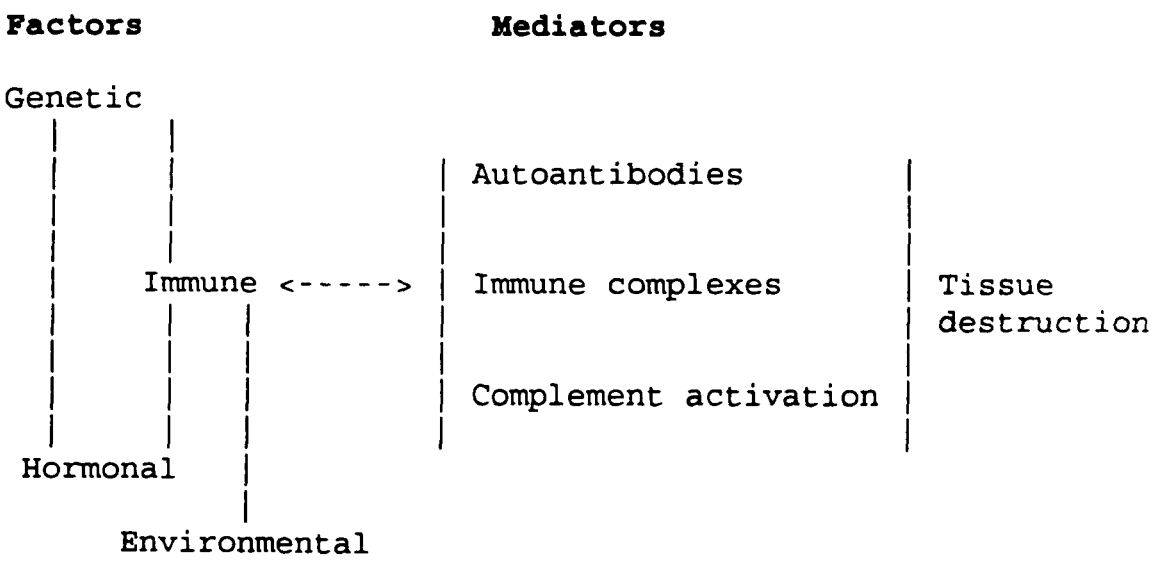
associated with autoimmune diseases, map within the MHC of man, or HLA region. The MHC comprises of three functionally and polymorphic gene classes: classes I, II and III. Most of the MHC associations with SLE, the prototype of autoimmune diseases, involve class II (HLA-DR and DQ alleles) and class III alleles, especially null or deficiency alleles of the fourth component (C4) of complement (Bias et al., 1986; Goldstein and Arnett, 1987; Braun and Zachary, 1988; Arnett and Moulds, 1991; Deodhar, 1992).

Role of B-lymphocytes- Polyclonal B cell activation has been proposed as one possible mechanism that may be responsible for over activation of B-cells and production of autoantibodies in certain autoimmune disease, particularly SLE (Klinman et al., 1990; Steinberg et al., 1990; Steinberg, 1992). B lymphocytes are normally subjected to heavy and ongoing selection through their antigen specific Ig receptors. B-cell tolerance can be mediated by multiple mechanisms. Tolerance can lead to cell death or editing of antigen-receptor genes. Defects in B-cell tolerance may play a role in, or cause systemic autoimmunity (Nemazee, 1993). Zouali (1994) has proposed that hyperproduction of lupus associated autoantibodies arises through a two stage mechanism whereby a general activation of the multireactive preimmune B-cell repertoire precedes oligoclonal expansion of selected B-cell clonotypes. It has also been indicated

that precursor B cells for IgG anti-dS DNA response in SLE patients are similarly selected and expanded, as are precursor B-cells specific for any foreign antigen, which implies that IgG anti-ds DNA response in SLE patients is antigen driven (Schwab et al., 1994).

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a prototype human autoimmune disease that manifests itself with a variety of fascinating clinical and immunological features. Although the causes of SLE and autoimmunity in general, remain unknown, considerable evidence has accrued on the pathophysiologic mechanisms that lead to the failure of distinction between self and non-self. Various factors that could either directly or indirectly be involved in the etiology of autoimmunity have been suggested (Tsokos, 1992) and are given below -



SLE is a multisystem inflammatory disease characterized by diverse ANA specificities. In patients with this disorder ANA production is almost invariable; over 95% of patients have a positive fluorescent antinuclear antibody (FANA) test (Pisetsky, 1994). Table 2 lists common ANA specificities found in lupus, designating the frequency of the antibody and the structure and function of the antigens. These specificities can be divided into two groups- those unique to SLE and those found in other rheumatic diseases. Only anti-DNA and anti-Sm are unique to SLE and serve as criteria in disease classification. Despite the presence of antibodies to both ss and ds DNA, most clinical assays are designed to detect antibodies to ds DNA because of increased specificity for diagnosis (Pisetsky, 1993).

Anti-DNA antibodies are the most prominent serological abnormality of SLE and occur in patients as well as spontaneous animal models of lupus. The presence of anti-DNA antibodies in the serum of SLE patients has long been considered both as marker of, and pathologic factor in renal disease (Andres et al., 1970; Koffler et al., 1974). There are reports of serum anti-ds DNA antibody levels correlating with the severity of the renal disease in SLE (Wallace et al., 1993). Recently Okamura et al (1993) have shown a strong correlation between anti-ds DNA IgG levels and renal histology that was not evident for anti-ds DNA IgM or for

TABLE 2

Principal Antinuclear Antibodies in Systemic Lupus Erythematosus

Specificity	Target Antigen	Function	Frequency in SLE (%)
Native DNA	dsDNA	Genetic information	40
Denatured DNA	ssDNA	Genetic information	70
Histones	H1,H2A,H2B,H3,H4	Nucleosome structure	70
Sm	snRNP proteins B,B',D,E	Splicesome component, RNA processing	30
U1RNP	snRNP proteins A,C,70K	Splicesome component, RNA processing	32
SS-AR/Ro	60-kd and 52-kd protein,complexed with Y1-Y5 RNA	Unknown	35
SS-B/La	46-kd protein complexed with various RNA	Regulation of RNA polymerase 3 transcription	15
Ku	86-kd and 66-kd proteins	DNA binding	3
PCNA/cyclin	36-kd protein	Auxillary protein of DNA polymerase	10
Ribosomal RNP	38-kd,16-kd,and 15-kd phosphoproteins,associated with ribosomes	Protein synthesis	10

Source : Tan, E.M. (1989)

anti-single stranded DNA (anti ssDNA) antibodies of either isotype. Various serological findings suggest that lupus nephritis results from the deposition of DNA-anti-DNA complexes and subsequent complement mediated tissue damage (Emlen et al., 1986; Pisetsky, 1992).

It is evident that the development of antibodies *per se* even anti-DNA antibodies, does not automatically lead to autoimmune disease. Healthy individuals, notably, the elderly and especially relatives of patients with autoimmune disease may develop a range of autoantibodies without any adverse effect (Shoenfeld and Isenberg, 1989). This observation must be viewed in the context of anti-DNA antibodies. While, 20-30% of the healthy relatives of SLE patients demonstrate the presence of antibodies against single stranded DNA (Miles and Isenberg, 1993), antibodies to double stranded, or native, DNA virtually never occur in these relatives. Thus antibodies to ds DNA do seem to be particularly associated with SLE.

The question of a direct role of anti-ds DNA antibodies in the pathogenesis of murine lupus has been reexamined recently. Vlahakos et al. (1992a) have described the ability of murine monoclonal anti-ds DNA antibodies (derived from MRL/lpr and SNF1 lupus prone mouse strains) injected into healthy-strain mice to form immune deposits at distinct glomerular and vascular sites. Taking lead from the work of

Shmiedeke et al. (1989), who showed that histones can bind with high affinity to glomerular basement membrane (GBM), Brinkman et al. (1990) and Termaat et al. (1992) have reported that anti-DNA antibodies bind to glomerulus via complexes of histones and DNA and, to a lesser extent via DNA alone. In another study, Vlahakos et al. (1992b) have provided evidence that murine monoclonal antibodies were able, *in vivo*, to penetrate cells and interfere sufficiently with their normal function as to contribute to their pathologic abnormalities. However, a very small percentage (5 out of 30) of these antibodies were able to do this, which suggests that direct anti-DNA antibody penetration is unlikely to be a significant pathologic process in SLE patients. The question of antibody penetration now goes back to some 15 years (Alarcon et al., 1978), since the original suggestion that anti-RNP antibodies might possess this ability. However, with rare exceptions (Okudaira et al., 1987), no other group has convincingly described this phenomenon. Recent studies have again reiterated direct association between anti-RNP autoantibodies and severity of lupus nephritis (Guialis et al., 1994; Reichlin et al., 1994).

In other studies of murine lupus, IgM anti-ssDNA antibody derived genes have been expressed in healthy strains of mice (Erikson et al., 1991). It was shown that

although many B-cells in these animals were able to express anti-DNA immunoglobulin on their surface, the cells were anergic-perhaps as a result of having been exposed to antigen. The healthy strain mice were able to control these potentially disease associated antibodies. Tsao et al (1992) have described another transgenic model utilising H and L chain genes from a hybridoma secreting an IgG monoclonal anti-DNA antibody. Many of the B-lymphocytes in this transgenic model expressed endogenous IgM and some expressed low levels of transgene-derived IgG, on cell membrane. As in the initial model, the transgenic animals did not develop major manifestations of lupus. However, these animals were found to have elevated levels of IgG anti-DNA antibodies in their serum and a minor degree of nephritis was noted. Interestingly, immunizing these animals with DNA increased the anti-DNA levels.

These results fall short of explaining the origins of human lupus in general or anti-ds DNA antibodies in particular. Three main concepts on the origin of human lupus have emerged from various experiments performed on genetically predisposed models. The first concept proposes that systemic autoimmune disease is the result of polyclonal B-cell activation (Klinman and Steinberg, 1987). The second suggests that autoreactive clones are the result of antigen-driven specific stimulation (Hardin, 1986). The third

envisages a two stage development, incorporating elements of both polyclonal activation and an antigen driven response (Zouali, 1992). Based on evidence available from human studies, the third view is considered to be the most valid explanation (Isenberg et al., 1994).

Mammalian DNA is non-immunogenic in a variety of animal species tested (Schwartz and Stollar,1985). However, sequence analysis of anti-DNA antibodies shows features of antigenic selection. In contrast to mammalian DNA, bacterial DNA can induce a good antibody response (Gilkeson et al., 1989). It has been noted that anti-DNA antibodies may be very similar in structure to anti-bacterial antibodies (Hahn and Tsao, 1993). This suggests that anti-dsDNA might arise from stimulation with foreign, rather than self DNA. Recently, Krishnan and Marion (1993) have shown that monoclonals, though a small number, generated against a complex of native DNA and a synthetic DNA binding protein shared DNA specificities and V region structures with anti-DNA monoclonals derived from lupus prone NZB/NZW mice. Another possible explanation for the presence of these antibodies is that DNA linked to histones, abundantly found in nucleosomes may be a stimulating antigen (Ehrenstein et al., 1993).

It has also been suggested that activated phagocytic cells can release highly reactive oxygen species (ROS) which

can penetrate cell membranes, interact with nuclear DNA and cause the release of altered DNA, which in turn, could stimulate anti-DNA antibodies (Gordon et al., 1990; Alam et al., 1993).

Anti-DNA antibodies are polyspecific showing enormous cross-reactivity. The nature of epitopes that are bound by anti-DNA antibodies is only partially elucidated. A range of specificities is attributed to these immunoglobulins. Monoclonal antibodies and sera from SLE patients and lupus prone animal models show that reactivity may be greater towards denatured forms of DNA (Stollar et al., 1986). Anti-DNA antibodies may react directly with nucleosides and nucleotides, with some antibodies exhibiting marked preference for particular bases/base pairs. Thus reactivity with poly (dA-dT), poly(dG-dC), poly (dA), poly(dT), poly (d[BrU]) has been reported (Tron et al, 1980; Gripenberg et al, 1981; Hahn and Ebling, 1984; Gibson et al, 1985; Stollar et al., 1986). Poly (ADP-ribose), a polymer that is produced in response to certain forms of DNA damage has also been reported for reactivity with anti-DNA antibodies (Dudeney et al, 1986). Additional specificities include other forms such as ssRNA, dsRNA, triple helical RNA, RNA-DNA hybrids (Karounos et al, 1988). SLE autoantibodies have also been reported to recognize multiple conformations of DNA, e.g. left handed DNA (Z-form), DNA-psoralen photoadduct, DNA-

lysine photoadduct, hydroxyl radical modified DNA, DNA-8-methoxypsoralen photoadduct, estradiol-albumin-DNA conjugate (Sehgal and Ali, 1990; Hasan and Ali; 1990; Ali et al., 1991; Hasan et al., 1991; Alam and Ali, 1992; Alam et al., 1992; Ara et al., 1992; Ara and Ali, 1992; 1993; Arjumand and Ali, 1994; Moinuddin and Ali, 1994). Arif et al. (1994) have reported human autoantibody binding with polylysine-polyglutamate complex. Given that anti-DNA antibodies may exhibit polyreactivity, it is believed that at least some of the epitopes bound by these autoantibodies must be present in sugar phosphate backbone common to all polynucleotides (Alam et al., 1995).

Role of Steroids in SLE

The basic question on the origin of anti-DNA antibodies in autoimmune diseases like systemic lupus erythematosus (SLE) has not been answered until now. SLE is a chronic debilitating disease which primarily affects women of child-bearing age. A role for estrogens in the pathogenesis of SLE has long been suspected (Rose & Pittsbury, 1944; Tumulty, 1954; Chapel & Burns, 1971; Fine et al, 1981). DNA against which most of the antibodies are detected in SLE and other autoimmune diseases is no longer regarded as the antigen initiating the disease because immunization with DNA does not induce disease like SLE. Female predominance of SLE is well documented but the mechanism(s) by which estrogen

exerts its effects on autoimmune diseases is not clearly elucidated. Recently, it has been shown that estrogen augments autoantibody formation in normal mice by a direct stimulatory effect on $CD5^+$ B cells, known to be responsible for autoantibody production (Ahmad et al, 1989). Epidemiological studies (Masi and Medsgar, 1979) have shown an incidence of SLE in black women of approximately 1:250 and 1:2000 overall. SLE is nine times more common in women and fifteen times more common during child bearing years. Peak onset is between 16 and 25 years of age. The predominance of females among patients with systemic lupus erythematosus (SLE) and frequent exacerbation of disease during pregnancy (Jungers et al, 1982a) or following oral contraceptive therapy (Jungers et al, 1982b) strongly suggest that sex hormones influence disease activity. Patients with Kleinfelter's syndrome, i.e. males with 47 chromosomes are hyperestrogenic and have an increased incidence of SLE (Stern et al, 1977). Recent studies have revealed an abnormal pattern of estrogen metabolism among SLE patients. It has also been reported that an excessive level of testosterone oxidation at the C17 level is present in female SLE patients compared with normal women (Lahita et al, 1983). Plasma androgen levels have been found to be normal in male SLE patients (Stahl and Decker, 1978). However, a significant reduction in all plasma androgen

levels has been observed in SLE patients (Jungers et al, 1982c) either in acute phase of disease prior to any corticosteroid therapy or after therapeutic remission. Low plasma testosterone levels in women with SLE have also been confirmed (Lahita et al, 1983).

The ability of sex hormones to modulate murine lupus was revealed more than 10 years ago when Talal and his colleagues reported that castration of male NZB/NZW (B/W) mice eliminated a protective factor and resulted in enhanced autoimmune disease expression and death (Roubinian et al, 1977). It was then demonstrated by implanting 5-α-dihydroxytestosterone (DHT) in female B/W mice that the protective factor was male hormone (Roubinian et al, 1978). A significant therapeutic effect could be achieved even if DHT treatment was delayed until six months of age (Roubinian et al, 1979) when female B/W mice were already dying of SLE. These experiments demonstrated that, female sex hormones accelerate autoimmunity, whereas male sex hormones suppress it. It is now known that sex hormones modulate the normal immune response and therefore, their immunologic effects are physiological (Ahmad et al, 1985; Ahmad and Talal, 1988). In (NZB/NZW) F1 (NZB/W) mice, a murine model for human SLE, females develop an earlier and more severe disease compared to males (Howie and Helyer, 1968). In several studies it has been suggested that estrogen is an accelerator of the lupus

disease of NZB/W mice whereas testosterone has protective properties (Roubinian et al, 1978; Steinberg et al, 1979; Siiteri et al, 1980). Also, MRL 1pr/1pr (MRL/1) mice, spontaneously developing a rapidly progressive autoimmune disease, constitute a model for human SLE. Immune complex mediated glomerulonephritis, massive lymph node proliferation, vasculitis, arthritis and production of a variety of autoantibodies are some hallmarks of lupus disease in MRL/1 mice (Andrews et al, 1978; Theofilopoulos and Dixon, 1981). The mean life span of male and female MRL/1 mice is 5.1 and 4 months respectively (Murphy and Roths, 1978). Steinberg et al (1980) demonstrated that administration of testosterone to MRL/1 mice resulted in a less severe disease.

SLE patients, as well as B/W mice, demonstrate a delayed clearance of particulate immune complexes. Female B/W mice exhibit this abnormality earlier than males. Furthermore, androgens suppress this defect in female mice. Estrogens, in contrast, accelerate this defect in male B/W mice. This suggests an important influence of sex steroid hormones on the clearance of immune complexes (Ahmad et al, 1985).

Antibodies against steroids have been raised by immunizing mice with the ligand conjugated steroids. Monoclonal antibody production against proteinic and steroid

hormones has also been reported (Kohler and Milstein, 1975). The feasibility of generating monoclonal antibodies against progesterone (P_4) and their characteristics have also been reported (Fantl et al, 1981; 1982; Wright et al, 1982; White et al, 1982; Fantl and Wang, 1984; Gupta et al, 1987). Antihormonal and antinuclear (ANA) responses have been reported by Sarah and colleagues (1987) from a population of female mice immunized with estradiol-albumin.

The target tissues for various classes of steroid hormones contain characteristic extranuclear "receptor" proteins which combine with the hormone and accompany it to its site of localization in the cell nucleus. Sex hormones can influence the immune system through multiple pathways. They bring about their effect on target cells by first interacting with specific steroid receptors. Sex-hormone receptors have been found not only in classical reproductive tissues but also in diverse unrelated body systems, including the immune system. The presence of sex hormone receptors in these non-classical sites may indirectly have a relevance to the immune system (Talal and Ahmad, 1987).

Sex hormones have specific effects on lymphocytes. It appears that T cells are primary targets of sex-hormone action (Ahmad et al, 1985; Ahmad and Talal, 1987). Regulatory T-cells appear to be more sensitive to sex-hormones. In humans, estrogen receptors have been

demonstrated on OKT-8 positive cells of peripheral blood which have supressor/cytotoxic activity. All the cells closely associated with lymphocytes which are important in the functioning of the immune system bear sex hormonal receptors. For example, sex-hormone receptors are present on the thymic epithelial cells (Ahmad et al, 1985). It has long been argued that thymus is the primary lymphoid gland through which sex hormones influence the immune responses. Sex hormones may interact with thymic epithelial cells to alter the thymic microenvironment and the release of thymic hormones which in turn profoundly influence the immune system (Talal & Ahmad, 1987).

Objective of the Present Study

The polyspecific nature of anti-DNA antibodies and the non-immunogenecity of native DNA (B-conformation) poses a puzzling question regarding the origin of these antibodies. Elevated levels of anti-DNA antibodies are found in active lupus and represent serological markers for diagnosis and prognosis. SLE is a chronic syndrome that primarily affects women, pointing towards the possible role of sex hormones in disease etiology. In this study two aspects were probed; One, the possibility of Z-DNA being the immunogenic trigger for the production of anti-DNA antibodies in SLE and second, the possible involvement of female sex hormone in lupus

etiopathogenesis. Z conformation was induced in native DNA as a result of bromination in high salt and interaction with polyamines. Small size DNA fragment was covalently linked to estradiol-albumin to see whether the female sex hormone (estradiol) in conjugation with DNA could form a possible antigenic trigger for SLE and whether this conjugate would be recognized by naturally occurring SLE anti-DNA antibodies.

Experimental

A. MATERIALS

Chemicals

Calf thymus DNA, bovine serum albumin, estradiol-albumin, ß-estradiol, xylene cyanol FF, ethidium bromide, anti-human and anti-rabbit IgG alkaline phosphatase conjugate, Freund's complete and incomplete adjuvants, coomassie Brilliant Blue G250 & R250, dialysis tubing (of varying diameter), poly-L-glutamic acid, poly-D-lysine, poly-L-lysine, spermine, spermidine, putrescine, millipore filter (0.45µm pore size), nuclease S1 and 4-methyl umbelliferyl phosphate were purchased from Sigma Chemical Company, U.S.A. Dextran Blue 2000, DEAE Sephacel, Sephadex G-200, Sepharose 4B, protein A-Sepharose CL-4B, agarose (NA), micrococcal nuclease, poly(dG-dC).poly(dG-dC), poly(rG).poly(dC), were the products of Pharmacia Fine Chemicals, Sweden. Acrylamide, ammonium persulfate, bisacrylamide, N,N,N`,N`-tetraethyl methylene diamine and Tween 20 were obtained from Bio-Rad, U.S.A. Perchloric acid and sodium azide were from Ferak-Berlin, Germany. Acetaldehyde was a product by Fluka, Switzerland. p-nitrophenyl phosphate and Folin-ciocalteu reagent were purchased from Centre for Biochemical Technology, New Delhi. ELISA plates were from Nunc, Denmark and Dynatech, U.S.A. All other chemicals were of highest purity analytical grade procured from reputed Indian firms like, Qualigens, B.D.H. and Merck.

Equipment

Bausch and Lomb spectronic 20, Shimadzu UV-240 spectrophotometer equipped with a thermoprogrammer and controller, Shimadzu spectrofluorophotometer RF-540, ELISA Reader MR600 (Dynatech, USA), Jasco J-720 spectro-polarimeter, cooling centrifuge C-24 (Remi, India), Horizontal gel electrophoresis assembly GNA-100 (Pharmacia, Sweden), desk top microfuge RM-12C (Remi, India) and UV-fluorescent table TF-20M (Vilber Lourmat, France) were the major equipments used in this study.

Collection of Sera

The normal human sera were collected from healthy subjects without evidence of SLE or other autoimmune disorders. Sera of patients with systemic lupus erythematosus (SLE) were obtained from the patients admitted to the Medical College Hospital of A.M.U. and from the patients at the outdoor Medicine clinic of A.I.I.M.S., New Delhi. The SLE sera were obtained from the patients who had elevated levels of anti-DNA antibodies and met the revised criteria for the American Rheumatism Association (ARA) for the diagnosis of the disease (Tan et al, 1982). All serum samples were heat decomplemented at 56°C for 30 min. After decomplementation, the sera were stored at -20°C with 0.02 percent sodium azide as preservative.

B. METHODS

Purification of DNA

Commercially available, highly polymerized calf thymus DNA was purified free of proteins and single stranded regions (Ali et al, 1985). Solutions of DNA (2 mg/ml) in 0.1 SSC buffer (0.015 M Na-citrate, 0.15 M sodium chloride, pH 7.3) were mixed with equal volume of chloroform-isoamyl alcohol (24:1) in a stoppered measuring cylinder and shaken gently for 1 hr. The DNA present in the aqueous layer was separated from organic layer and again subjected to extraction. After extraction the DNA was precipitated with two volumes of cold 95% ethanol and collected on a glass rod. It was dried by pressing against the wall of the container. The DNA was dissolved in 30mM acetate buffer, pH 5.0 containing 30mM zinc chloride. The single stranded regions that might be present in the DNA were removed by treatment with nuclease S1 (200 units/mg of DNA at 37°C for 30 minutes). The reaction was terminated by adding one-tenth volume of 0.2 M EDTA pH 8.0. The purified DNA was extracted twice with chloroform-isoamyl alcohol and reprecipitated with cold 95% ethanol. The DNA precipitate was dried and dissolved in the required buffer.

DNA Estimation by Burton Method

Colorimetric estimation of DNA was carried out according to Burton (1956) using diphenylamine.

Diphenylamine reagent

The reagent was prepared immediately before use by dissolving 750 mg of recrystallized diphenylamine in 50 ml of glacial acetic acid containing 0.75 ml of concentrated sulphuric acid.

Assay procedure

Varying amount of DNA in 1.0 ml was mixed with 1 N perchloric acid and incubated at 70°C in a water bath for 15 minutes. One hundred µl of 5.43 mM acetaldehyde was added followed by 2.0 ml of diphenylamine reagent. The tubes were vortexed and allowed to stand at room temperature for 16-20 hr for color development. Absorbance was recorded at 600 nm. The DNA concentration in the unknown sample was determined from the standard plot of purified calf thymus DNA (Fig. 1).

Estimation of Protein

Protein was estimated by the method of Lowry et al. (1951) and Bradford (1976).

Protein Estimation by Folins-Phenol Reagent

This method of protein estimation utilizes alkali (to maintain high pH), Cu^{2+} ions (to chelate proteins) and tartarate (to keep the Cu^{2+} ions at high pH).

(a) Folin-ciocalteu reagent

The reagent was purchased from Centre for Biochemical

Technology, New Delhi (India). It was diluted with distilled water in the ratio of 1:4 before use.

(b) Alkaline copper reagent

The components of alkaline copper reagent were

(i) Two percent sodium carbonate in 0.1 M sodium hydroxide.

(ii) 0.5 percent copper sulphate in 1.0 percent sodium potassium tartarate.

The working reagent was prepared afresh by mixing components (i) and (ii) in the ratio of 50:1.

(c) Procedure

To 1.0 ml of protein sample was added 5.0 ml of alkaline copper reagent, the contents were mixed and left at room temperature for 10 minutes. Then 1.0 ml of Folin-ciocalteu reagent was added and after 30 minutes the absorbance was monitored at 660 nm. The protein content of unknown samples was determined from a standard plot of bovine serum albumin (Fig. 1).

Protein Estimation by Bradford Method

This colorimetric assay is performed under acidic condition. The color change occurs when coomassie Brilliant Blue G250 binds strongly to protein hydrophobically and at positively charged groups. In the environment of these positively charged groups, protonation is suppressed and a

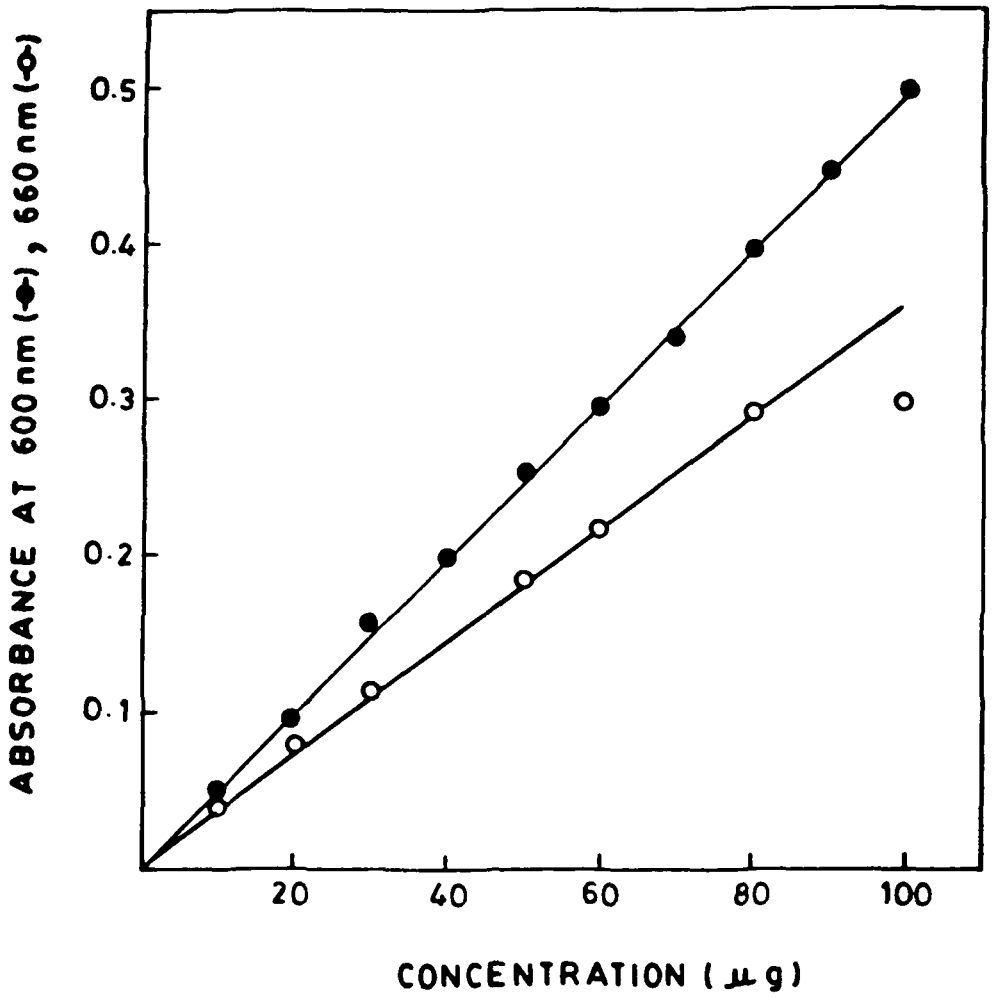


Fig.1. Standard plot for the colorimetric estimation of DNA (—●—) and protein (—○—).

blue color is observed (λ max = 596 nm).

(a) Dye preparation

One hundred milligram of coomassie Brilliant Blue G 250 was dissolved thoroughly in 50 ml of 95% ethanol. To this solution was added 100 ml of 85% (v/v) orthophosphoric acid. The resulting solution was diluted to a final volume of 1 liter. It was filtered before use.

(b) Protein assay

Solution containing 10-100 µg protein in a volume of upto 0.1 ml was pipetted into test tubes. The volume was adjusted to 1.0 ml with appropriate buffer. Five milliliter of dye solution was added and the contents were mixed by vortexing. The absorbance was read at 595 nm within an hour against a reagent blank prepared from 0.1 ml of buffer and 5.0 ml of dye solution.

Micrococcal Nuclease Digestion of Native Calf Thymus DNA

Purified calf thymus DNA (2 mg/ml) in 0.006 M Tris, 0.1 M sodium chloride, 0.002 M calcium chloride, pH 8.0 was digested with micrococcal nuclease (40 units/mg DNA) for 3.0 minutes at 37°C. The reaction was stopped by adding one-tenth volume of 0.2 M EDTA, pH 8.0. The sample, after precipitation with double distilled cold ethanol, was subjected to nuclease S1 digestion (200 units/mg DNA) for 30

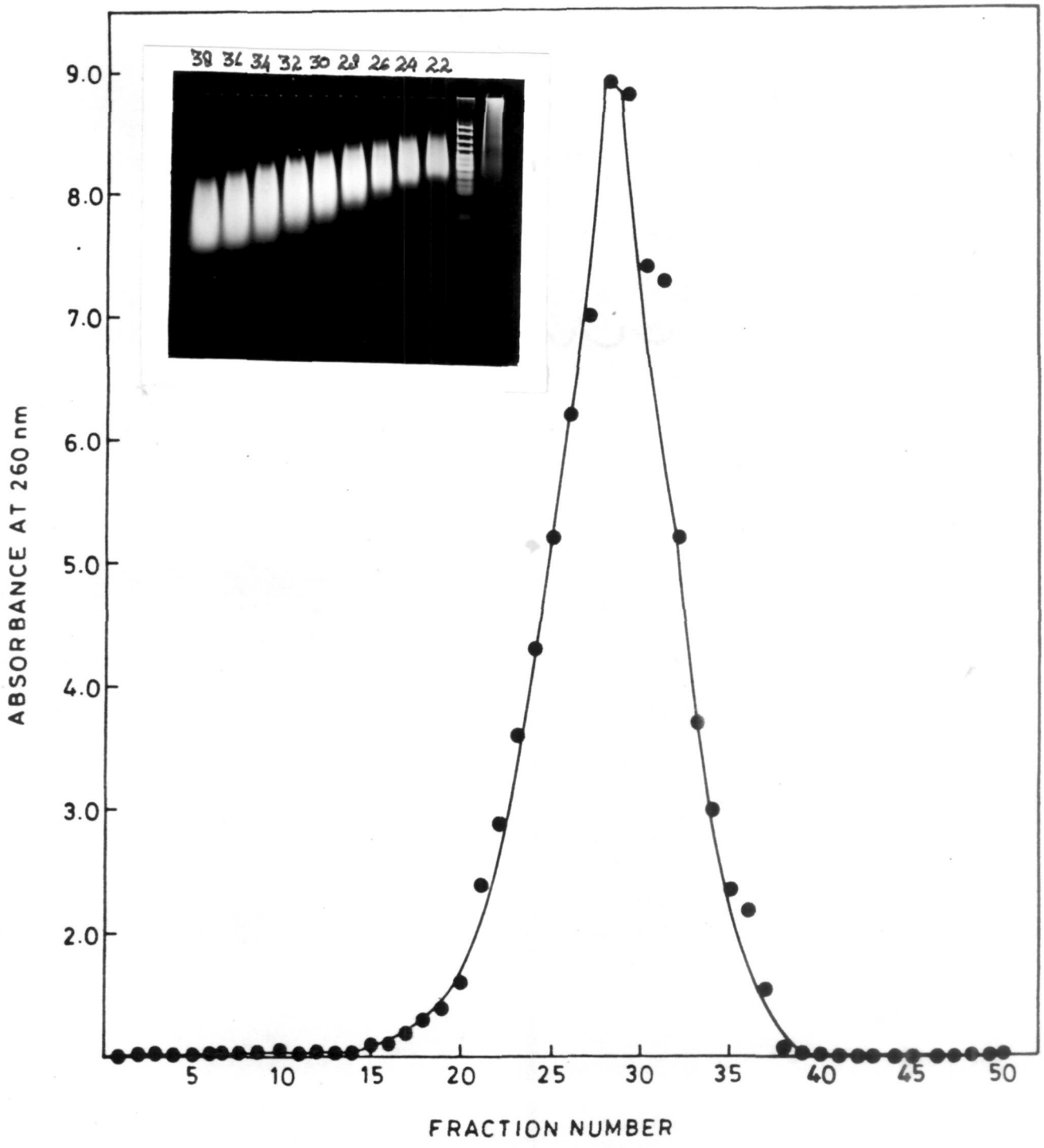


Fig.2. Elution profile of micrococcal nuclease digested native calf thymus DNA on Sepharose 4B gel-filtration column.

Inset: Polyacrylamide slab gel electrophoresis pattern of alternate fractions from 22 to 38 alongwith EcoRI and Hind III digested Lambda DNA as marker.

minutes at 37°C (Ali et al, 1985). The sample was extracted twice with chloroform-isoamyl alcohol and reprecipitated with cold ethanol. It was dissolved in TBS (0.01M Tris, 0.15 M sodium chloride, pH 8.0) and passed through a Sepharose 4B column (2cm x 44cm) previously equilibrated with the same buffer. Fractions of 4.0 ml were collected and monitored for DNA content at 260 nm, using 1.0 O.D at 260 nm =50 µg ds DNA/ml (Fig. 2). The size of the DNA fragments was analyzed by polyacrylamide gel electrophoresis without stacking gel using lambda DNA digest of Eco RI and Hind III as size marker.

Modification of DNA

(a) Bromination of DNA

Purified native calf thymus DNA was brominated under high and low salt conditions as described by Lafer et al (1981). High purity distilled water was saturated with liquid bromine. Native DNA was treated for 1 hr with 4 M NaCl in citrate buffer (0.02M sodium citrate, pH 7.2 containing 0.001 M EDTA) and used at a final concentration of 100 µg/ml. The same amount of DNA in PBS (0.01 M phosphate buffer, pH 7.4 containing 0.15 M NaCl) served as low salt control sample. The bromine water was added to DNA in 1:4 ratio and kept at room temperature for 30 minutes. After bromination, the samples were extensively dialysed against TBS, pH 7.4 to remove excess bromine. Native DNA,

brominated in high and low salt solutions were designated as Br4 and BrN respectively. Absorbance value of native and brominated DNA was recorded at 260, 280, 294 and 296 nm against TBS, pH 7.4. The average of absorbance at 294 and 296 nm divided by absorbance at 260 nm was termed as "absorbance ratio". The UV absorption spectra of low and high salt brominated DNA were recorded against buffer (TBS, 7.4) and compared with the control spectrum obtained with same concentration of DNA solution. While recording the UV difference spectra of Br-native DNA, the same concentration of native DNA served as control. The native and brominated DNA were also subjected to circular dichroism measurements. The baseline of the instrument was adjusted with the buffer.

(b) DNA- Polyamine interaction

Purified calf thymus DNA was first dialyzed against cacodylate buffer (0.001 M sodium cacodylate, 0.05 M NaCl, 0.00015 M EDTA, pH 7.4). The nucleic acid was then taken into cacodylate buffer but without EDTA. Stock polyamines solution were also prepared in the same buffer. The DNA was mixed with varying concentrations of different polyamines and incubated for 30 minutes at room temperature. Double stranded poly.(dG-dC) was also complexed with polyamine in the similar fashion. Solutions of nucleic acids without polyamine formed the control sample. For each concentration

of polyamines, triplicate sets of polyamine- nucleic acid were prepared.

(c) Preparation of DNA-BSA conjugates

Glutaraldehyde, a bifunctional cross linking reagent that links two compounds primarily through their amino groups was used for coupling DNA to BSA using the single step method of Reichlin (1980) with a slight modification. To 1.0 ml solution of fractionated DNA (average size 200 bp) from a stock of 1 mg/ml in PBS, pH 7.2 was added 30 µL of BSA from a 100 mg/ml stock in PBS. Then 1 ml of 0.2 % glutaraldehyde solution was slowly added with constant shaking and the mixture was incubated at room temperature for 1 hr. The reaction was stopped by adding glycine (in PBS, pH 7.2) to a final concentration of 200 mM followed by stirring for 1 hr at room temperature. The separation from unlinked constituents was achieved by gel exclusion on Sephadex G-200.

(d) Preparation of Estradiol-Albumin-DNA (E_2-BSA-DNA) conjugates

One milliliter solution of DNA (1 mg/ml stock in PBS, pH 7.2) was mixed with 3.0 ml of estradiol-albumin solution, (1 mg/ml in physiological saline). The contents were air-dried to 2.0 ml and 2.0 ml of 0.2 % glutaraldehyde solution was added and the mixture was left at room temperature for 1

hr. The reaction was stopped by adding glycine (in PBS, pH 7.2) to a final concentration of 200 mM. The separation of linked conjugates from unlinked entities was achieved by gel filtration on Sephadex G-200.

Separation of Linked Conjugates (DNA-BSA and E_2-BSA-DNA) From Unlinked Constituents

The separation was achieved by gel filtration on a Sephadex G-200 column (46 cm x 1.0cm) previously equilibrated with PBS, pH 7.2. Just after the completion of the reaction, the sample was loaded on the column and eluted with the equilibrating buffer. Fractions of 2.5 ml were collected and the amount of DNA and protein in each fraction was determined by the method of Burton and Lowry et al. respectively. Fractions containing both DNA and protein were pooled together and taken as DNA-BSA and E_2-BSA-DNA conjugates. These fractions were characterized by ultraviolet spectroscopy.

Absorption-Temperature Scan

Native and modified DNA samples were subjected to heat denaturation by an electronically controlled heating device (Hasan and Ali, 1990). All the samples were melted from 30°C to 95°C at a rate of 1.0°C/min after 10 minutes equilibration at 30°C. The change in absorbance at 260 nm was recorded with increasing temperature. Percent

denaturation was calculated using the following equation.

$$\text{Percent denaturation} = \frac{A_T - A_{30}}{A_F - A_{30}} \times 100$$

Where,

A_T is the sample absorbance at a temperature T°C.

A_F is the final absorbance at 95°C.

A_{30} is the initial absorbance at 30°C.

Thermodynamic Analysis of Modified/Unmodified Nucleic Acids

The thermal transition of nucleic acid from native to denatured state was characterized as a single variable, f_D, the fraction of nucleic acid in the denatured state. The percent loss in absorbance was used as experimental variable to follow the transition. At any given point along the transition curve, the observed percent change in absorbance was related to the fraction of nucleic acid in the denatured state, by the following expression-

$$f_D = \frac{(A)_{obs} - (A)_N}{(A)_D - (A)_N}$$

Where, $(A)_{obs}$, $(A)_N$ and $(A)_D$ represent the percent loss in absorbance in any observed, native and fully denatured states respectively. Since each experimental value of $(A)_{obs}$ will give unique value of $(A)_D$, the later was used to construct transition curves for the thermal denaturation of nucleic acids.

By assuming that unfolding of the strands of nucleic acid is a two state process due to the presence of AT and GC regions respectively, the initial and final states of thermal denaturation of nucleic acid were related to apparent equilibrium constant, defined as:

$$K_{app} = \frac{(A)_{obs} - (A)_N}{(A)_D - (A)_{obs}}$$

The free energy change, ΔG, for the thermal reaction (native double stranded conformation to single stranded conformation) was calculated by using the following relationship

$$\Delta G_D = -RT \ln K_{app}$$

Where, K_{app} is the apparent equilibrium constant, R is the gas constant (8.314 J/mol/deg) and T is the absolute temperature.

Time Course Kinetic Study of Thermal Energy Melting of Unmodified/Modified Nucleic Acids

The noncovalent/covalent modifications incurred in DNA as a consequence of interactions with spermine, protein and hormone- protein conjugate were also probed by kinetic of thermal melting.

Time course kinetic analysis of the helix coil transition was performed by the method of Warren et al. (1974) using the following first order rate equation

$$-\ln (Y_{fD(\infty)} - Y_{fD(t)}) = a+kt$$

Where $Y_{fD(\infty)}$ and $Y_{fD(t)}$ are the values for the fraction of nucleic acid in the denatured state at infinity (i.e. time reached at 95°C, where complete separation of the two strands occur) and at any time 't' (corresponding to its melting temperature).

The values of $-\ln (Y_{fD(\infty)} - Y_{fD(t)})$ versus time 't' were plotted and the apparent first order rate constant was determined from the slope.

Preparation of Heat Denatured DNA

Purified double stranded DNA was subjected to 20 minutes heating in a boiling water bath followed by immediate chilling on ice-NaCl mixture. Stoppered glass tubes were used to prevent evaporation of the solvent.

Immunization Schedule

Female rabbits were immunized with the following antigens emulsified with equal volume of Freund's complete/incomplete adjuvants as per the protocol described earlier (Hasan and Ali, 1990).

(a) 100 µg of estradiol-albumin.

(b) 50 µg of DNA linked to BSA.

(C) 50 µg of DNA linked to estradiol-albumin.

The first injection was given in complete adjuvant and for the rest four, Freund's incomplete adjuvant was used.

The volume of each injection was kept at 1.0 ml. Blood was collected by cardiac puncture after the fourth booster and the antibody activity was checked in the immune serum. Preimmune serum was obtained from the blood collected before immunization.

Purification and Isolation of IgG

(a) Preparation of crude immunoglobulins

Heat decomplemented serum was precipitated by saturated ammonium sulfate solution. To 6.5 ml of the serum was added 3.5 ml of 100 percent saturated ammonium sulfate solution with gentle shaking in cold and the mixture was left for 1 hr at 4°C for the complete precipitation of immunoglobulins. The precipitate was recovered by centrifugation and washed thrice with 35 percent ammonium sulfate solution. The washed precipitate was dissolved in and dialyzed against 10 mM phosphate buffer, pH 8.0.

(b) DEAE Sephacel chromatography

Crude immunoglobulins were loaded on a DEAE Sephacel column (1.5cm x 22cm) previously equilibrated with 0.01M phosphate buffer, pH 8.0. The unbound proteins were washed off with the equilibration buffer and the bound material was eluted with a linear ionic gradient of 0.01M-0.3M phosphate buffer pH 8.0. Fractions of 4.0 ml were collected at a flow rate of 20 ml/hr. These fractions were monitored for their

absorbance at 251 nm, 278 nm and 280 nm. Peak fractions with A_{278}/A_{251} ratio of 2.5 or more were pooled as IgG. The homogeneity was checked by polyacrylamide gel electrophoresis, wherein a single band confirmed that the IgG was homogenous (Fig. 3).

Immunoaffinity Purification of Antibodies

(a) CNBr activation of Sepharose 4B

15 ml of supplied Sepharose 4B was suspended in distilled water, and then washed with 300 ml of cold distilled water on a sintered glass funnel (porosity G-2). Moist gel (about 10g) in 10ml of 2.0 M sodium carbonate was kept in ice-NaCl bath placed on a magnetic stirrer. One gram cyanogen bromide in 0.8 ml acetonitrile was slowly added to the gel with gentle stirring. The reaction was allowed for 12 min and after that the reaction product was filtered on a sintered glass funnel, washed with 400 ml of cold 0.01 M sodium bicarbonate and resuspended in 10 ml of the same buffer. The unreacted CNBr was treated with ferrous sulphate to convert it into harmless ferrocyanide. All steps were carried out in a fume-hood chamber (Ali, 1984).

(b) Poly-L-lysine coupling to activated Sepharose 4B

The method described by Wilchek (1973) was followed. Immediately after CNBr activation of gel, equal volume of poly-L-lysine (100 mg in 10 ml of 0.1M sodium bicarbonate)

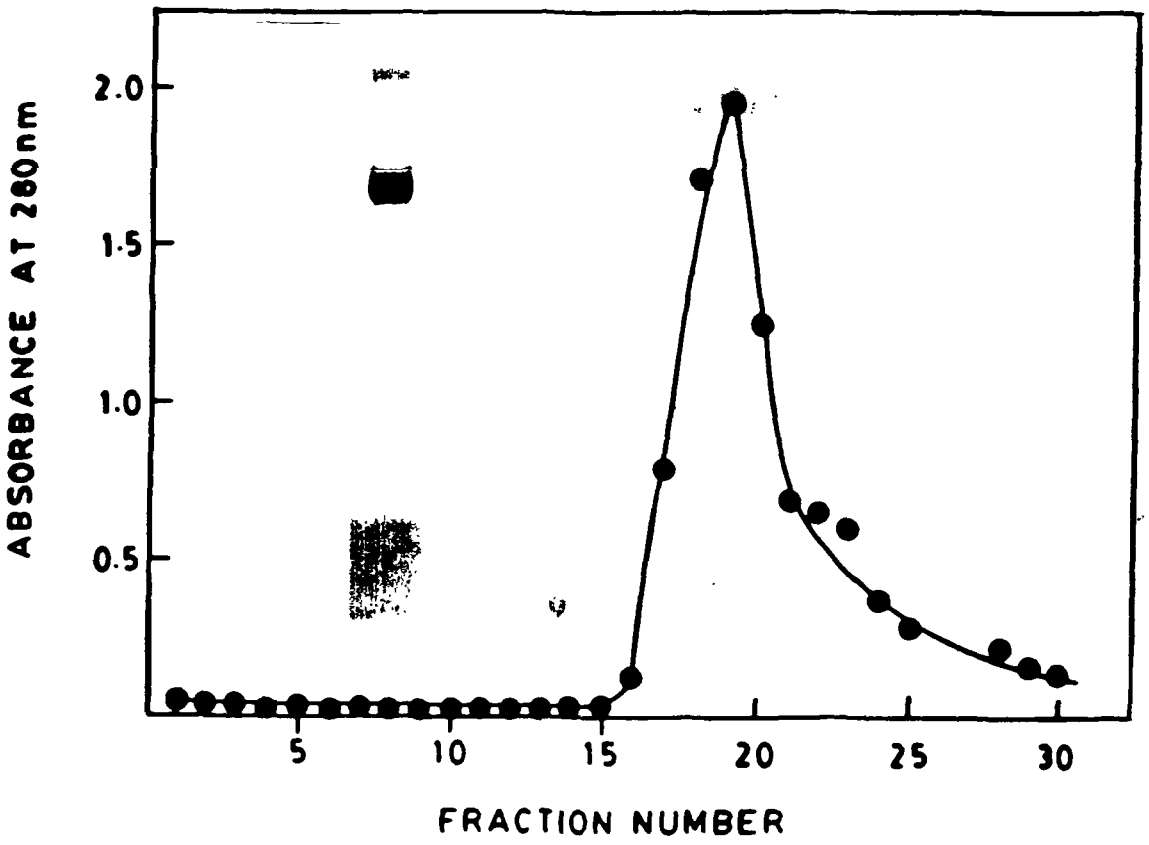


Fig.3. Isolation of IgG from an immune serum by DEAE Sephacel chromatography. Thirty five percent saturated ammonium sulphate precipitated serum proteins were loaded onto the column. Peak fractions were pooled as IgG.

Inset: Polyacrylamide gel electrophoretic mobility of isolated IgG on 7.5% gel.

was added to the activated gel and kept at 4°C for 12 hr with slow stirring. The buffer was drained out and the gel was washed successively with 100 ml each of cold (i) distilled water, (ii) 0.1N HCl, (iii) 0.1 M sodium bicarbonate and (iv) distilled water till neutral. Finally the gel was suspended in 40 ml of 0.15 M acetate buffer, pH 4.5. The extent of poly-L-lysine depletion due to its covalent coupling with activated Sepharose 4B was calculated by TNBS estimation of lysine in the drained effluent as described by Habeeb (1966).

(c) Affinity isolation of antibodies

DNA-[polylysyl-Sepharose 4B] was prepared as described by Nicotra et al, (1982) with a little modification. Previously prepared 20 ml polylysyl-Sepharose 4B was packed and equilibrated with 25 ml acetate buffer in a mini column (1.5cm x 5cm). Purified DNA (12.5 ml of 100 µg/ml) in acetate buffer was passed through the gel and unbound DNA was washed with PBS, pH 7.4 (Estradiol-albumin-DNA was used instead of native DNA for the affinity purification of antibodies raised against E_2-BSA-DNA). Heat decomplemented serum dialyzed against and diluted (1:10) with PBS was passed through the column. Unbound proteins were washed with PBS and the bound antibodies were eluted with linear ionic strength gradient of 0.15-3.0 M sodium chloride in 0.01 M phosphate buffer, pH 7.4. Fallthrough fractions were

monitored at 251 nm, 260 nm, 278 nm and 280 nm.

(d) Regeneration of affinity column

The column was regenerated several times by washing successively with 50 ml each of the following: (i) distilled water, (ii) 0.1 N HCl, (iii) 0.1 M sodium bicarbonate, (iv) distilled water till neutral.

Gel Electrophoresis

(a) Polyacrylamide slab gel electrophoresis for proteins

Polyacrylamide slab gel electrophoresis was performed under non-denaturing conditions as described by Laemmli (1970). The following stock solutions were prepared.

(i)	Stacking gel buffer	:	0.5M Tris-HCl, pH 6.8
(ii)	Resolving gel buffer	:	3.0M Tris-HCl, pH 8.8
(iii)	Reservoir buffer	:	0.025M Tris, 0.192M glycine pH 8.3
(iv)	Acrylamide-bisacrylamide	:	(30 : 0.8)

Thirty gram acrylamide and 0.8g bisacrylamide were dissolved in distilled water to a total volume of 100 ml. The solution was stored at 4°C in an amber colored bottle.

(b) PAGE procedure

The glass plates (19cm x 16cm) were soaked in chromic acid and thoroughly washed with tap water followed by one rinse with distilled water and ethanol. Finally the plates

were dried and sealed with 1% agarose with 1.5 mm thick spacer separating them. 7.5% separating gel was layered with 2.5% stacking gel according to the recipe given below (all volumes are in ml).

Solutions	**Stacking gel**	**Resolving gel**
Acrylamide-bisacrylamide	2.5	7.5
Stacking gel buffer	5.0	-
Resolving gel buffer	-	3.75
Distilled water	10.0	17.25
1.5% Ammonium persulphate	1.5	1.5
TEMED	0.02	0.02

The reagents were mixed and poured between glass plates. The gel was allowed to polymerize at room temperature and when polymerization was completely achieved, the protein sample (25-50µg) containing 10% sucrose and 0.002% bromophenol blue was applied and electrophoresis was carried out at 70 volts for 6-8 hr. It was left for overnight staining in 0.1% coomassie Brilliant Blue (R250) mixed with 25% iso-propyl alcohol and 10% glacial acetic acid. Destaining was carried out by a mixture of 30% methanol having 10% glacial acetic acid.

(c) Slab gel electrophoresis for native DNA

The procedure of Sealey and Southern (1985) was

followed for electrophoresis of DNA. The following buffers were used for the purpose.

(i) 40% acrylamide in distilled water

(ii) 2% bisacrylamide in distilled water

(iii) Resolving gel buffer : 0.9 M Tris-borate, pH 8.3 containing 0.025M EDTA.

(iv) Electrode buffer: 0.09 M Tris-borate, pH 8.3 containing 0.0025 M EDTA.

Recipe for 7.5% DNA-PAGE (all volumes in ml)

Solutions	**Resolving gel**	**Stacking gel**
40% Acrylamide	7.5	1.25
2% bisacrylamide	7.5	1.25
Gel buffer	4.0	1.0
Distilled Water	21.0	6.5
Ammonium persulphate	50 mg	15 mg
TEMED	0.025	0.025

The DNA sample (0.5 -1.0 µg) was mixed with one - tenth volume of dye (30% Ficoll, 0.025% xylene cyanol FF in gel buffer) and electrophoresed for 6-8 hr at 70 volts. The gel was stained with ethidium bromide (1 µg/ml) and DNA bands were visualised under UV light.

(d) Agarose gel electrophoresis of DNA

Agarose gel was prepared by dissolving agarose NA in

electrophoresis buffer (0.04 M Tris-acetate, pH 8.0 containing 0.002 M EDTA). Molten agarose was poured on the gel tray and allowed to solidify for 1 hr at room temperature. The DNA samples were electrophoresed for 2-4 hr at 30mA in the same buffer. The gel was stained with ethidium bromide.

(e) Gel retardation assay

The antigen-antibody interaction was analyzed by band shift assay. A constant amount of antigen (0.2 µg) was mixed with varying amount of antibody (0-20 µg) in a total volume of 20-30 µl in 0.01M sodium phosphate buffer, pH 7.4 containing 0.15M sodium chloride. The complex was incubated for 2 hr at room temperature and overnight at 4°C. At the end of incubation, 2 µl dye solution was added to each tube and the complex was electrophoresed on agarose gel for 2 hr at 30 mA using Tris-acetate EDTA (pH 8.0) buffer. On completion of electrophoresis, gels were stained with ethidium bromide and photographed under UV illumination. Appropriate controls were also run simultaneously.

Detection and Quantitation of Antibodies

(a) Immunodiffusion (ID)

ID was carried out by ouchterlony double diffusion system using glass petridishes. Six ml of 0.4% molten agarose in PBS, pH 7.4 containing 0.1% sodium azide was

poured onto glass petridishes and allowed to solidify at room temperature and then kept at 4°C for four hours. Wells, each 5mm in diameter and separated by a 8 mm distance were cut into the hardened gel by a cork borer. Antigen and antibodies were placed in the wells and the reaction was run for 48-72 hr at room temperature.The petriplates were washed with 5.0% Na-citrate for two hours to remove nonspecific precipitin lines, if any.

(b) Counterimmuno-electrophoresis (CIE)

The method of Kurata and Tan (1976) was followed for performing CIE. Molten agarose (0.6%) in 0.025M barbital buffer, pH 8.4 containing 0.1% sodium azide was poured onto 2.5 mm thick glass slides (2.5cm x 7.5cm) and allowed to harden at room temperature and then at 4°C. Wells, each 3mm in diameter were filled with antigen (in the cathodal well) and antibodies (in the anodal well). Electrophoresis was performed for 30 min in 0.05M barbital buffer, pH 8.4 under 3-4 mA current per slide. Non specific precipitin lines were removed by 5% sodium citrate solution washing.

(c) Enzyme-linked immunosorbent assay (ELISA)

(i) Buffers and reagents

Tris buffer saline (TBS) : 0.01M Tris
0.15M NaCl
pH 7.4

Tris buffer saline Tween-20 (TBS-T)	: 0.02 M Tris 0.144 M NaCl 0.00268 M KCl pH 7.4 containing 500 µl Tween-20
Bicarbonate buffer	: 0.015 M Sodium carbonate, 0.035 M Sodium bicarbonate, pH 9.6
2-amino-2 methyl-1-propanol (AMP) buffer	: 0.15 M AMP Containing 0.003 M $MgCl_2$, pH 10.3
Substrates	: (a) 500 ug p-nitrophenyl phosphate per ml of bicarbonate buffer containing 0.002 M $MgCl_2$. (b) 4-methyl umbelliferyl phosphate (4 MU-P) in 0.15 M AMP buffer.

(ii) ELISA procedure

The procedure of Aotsuka et al. (1979) with slight modification (Alam and Ali 1992) was used for detection and quantitation of antibodies formed against E_2-BSA, E_2-BSA-DNA and DNA-BSA conjugate.

Polystyrene microtiter plates were coated with 100 ul of either of the three antigens, (E_2-BSA, E_2-BSA-DNA or DNA-BSA) in bicarbonate buffer at a concentration of 5 µg/ml for E_2-BSA and 2.5 µg/ml for the other two. The plates were incubated at 37°C for 2 hr followed by overnight incubation at 4°C. The plates were washed three times with TBS-T and the unoccupied sites were blocked by 150 µl/well of 1.5% BSA in TBS for six hours at room temperature (10% skimmed milk was used to block unoccupied sites when antibodies against DNA-BSA were used). The plates were washed twice with TBS-T

and the antibody (100 µl/well) to be tested, diluted in TBS was added to each well. After two hours incubation at 37°C and overnight at 4°C, the plates were washed four times with TBS-T. Bound antibodies were assayed by an appropriate anti-immunoglobulin alkaline phosphatase conjugate using p-nitrophenyl phosphate as substrate. The plates were incubated at 37°C for 30 min and the reaction was stopped by adding 100 ul of 3 M NaOH solution to each well. Each sample was coated in duplicate and the absorbance of each well was monitored at 410 nm. Results were expressed as a mean of A_{test}-$A_{control}$.

ELISA for Anti-DNA Antibodies

Polystyrene microtiter plates were incubated with 100 ul of poly D-lysine solution (50 µg/ml in distilled water). The plates were washed three times with TBS and coated with 100 µl of antigen (ds DNA) at a concentration of 2.5 µg/ml in TBS for two hours at 37°C followed by overnight incubation at 4°C. The plates were washed three times with TBS-T and saturated with 100 µl of poly L-glutamate (50 µg/ml in TBS) for two hours at room temperature. Rest of the steps were same as in the previous case.

ELISA for the Binding of SLE Autoantibodies to ß-Estradiol

ELISA was performed on 96 well flat bottom polystyrene plates with slight modification in the procedure described

earlier.

The plate was precoated with 100 µl/well poly-L-lysine (100 µg/ml in CO_3/HCO_3 buffer, pH 9.6) for 2 hr at 37°C. After three washings with TBS (10 mM Tris, 150 mM NaCl pH 7.4) test wells were coated with 100 µl ß-estradiol (2.5 µg/ml in double distilled ethanol). The plate was kept for 2 hr at 37°C and overnight at 4°C and then washed thrice with TBS-T (20 mM Tris, 150 mM NaCl, pH 7.4 containing 0.05% Tween-20). Hundred microlitre of poly-L-glutamate (50 µg/ml in TBS, pH 7.4) was coated to each of the antigen coated wells for 2 hr at 37°C. After three wash with TBS-T, the plate was filled with 150 µl of 1.5% TBS-BSA and incubated for 5 hr at 37°C. Antibody (100 µl/well) to be tested was added to each well in different dilutions. The plate was left for 2 hr at 37°C and overnight at 4°C. Bound antibodies were assayed with anti-human IgG alkaline phosphatase conjugate (1:1500 dilution in TBS) using p-nitrophenyl phosphate and 4MU-P (in 0.15 M AMP buffer) as substrate. The contents of each well were read at 410 nm on an automatic microplate reader for PNPP, and fluorometric measurements (using 4 MU-P as substrate) were made on RF-540 Shimadzu spectrofluorophotometer equipped with a DR-3 data recorder.

Inhibition ELISA

The antigen binding specificity of antibody was defined by competition-inhibition experiments (Hasan et al, 1991). Varying amounts of inhibitors were mixed with a constant amount of antiserum or IgG. The mixture was incubated for 2 hr at 37°C and 16-20 hr at 4°C. Instead of serum, the resulting immune complex was used in ELISA. The results were expressed as percent inhibition.

$$\% \text{ Inhibition} = 1 - \frac{A_{\text{inhibited}}}{A_{\text{uninhibited}}} \times 100$$

Results

Purified native calf thymus DNA was modified by (a) bromination under high and low salt conditions, (b) interaction with polyamines, (c) covalently linking DNA (average size 200 bp) to BSA and E_2-BSA respectively. The modified polymers were characterized by physico-chemical methods and their antigenecity was evaluated by immunization in experimental animals.

UV Absorption Properties of Brominated DNA

Native calf thymus DNA brominated in low (0.15 M NaCl) and high salt (4.0 M NaCl), when subjected to UV-spectroscopy under physiological conditions showed the characteristic hypochromicity at 260 nm with concomitant hyperchromicity at 295 nm compared to the spectrum of native DNA. The λmin (under high salt) was shifted from 230-242 nm, while no appreciable shift was observed in the case of low salt sample (Fig. 4a). The UV difference spectra of the above samples exhibited positive band peaking at around 300 nm. The effect was less pronounced in the DNA sample brominated in low salt. (Fig. 4b). The UV-absorption characteristics of native and brominated DNA sample under physiologically compatible conditions are listed in Table 3. The data show around 60% decrease in absorbance as a result of bromination in high salt, while only 27.5% decrease was observed for the low salt brominated polymer. The conformational changes in DNA were also evaluated by

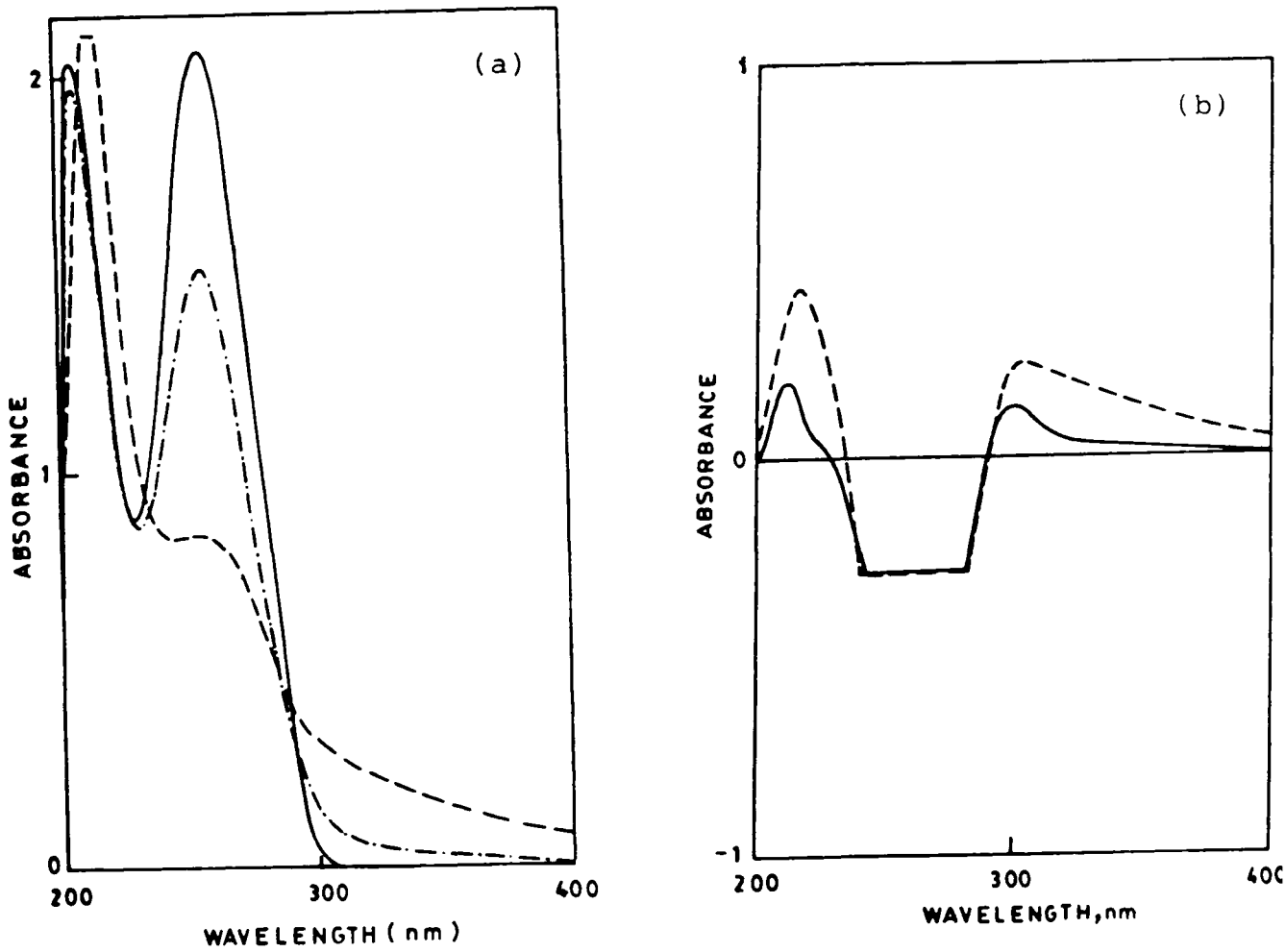


Fig.4. **(a)** Ultraviolet absorption spectra of native calf thymus DNA(——), brominated in 0.15M (-·-·-) and in 4.0M (----) NaCl.

(b) UV difference spectra of native DNA brominated in low (——) and high (-----) salt. Unbrominated DNA served as control.

quantitative measurements of absorbance at 260 nm and 295 nm. The absorbance ratio (A_{295}/A_{260}) was 0.130 for native DNA, 0.188 for native DNA brominated under physiological conditions of saline and 0.35 for the polymer brominated in high salt (Table 3). It could be noted here that the absorbance ratio (A_{295}/A_{260}) is 0.3 for Z-DNA (Thomas and Strobel, 1988).

Circular Dichroism of Native and Brominated DNA

Double stranded poly(dG-dC) undergoes B→Z transition in high salt. The circular dichroism (CD) of this polymer in high salt shows near inversion of the spectrum with a positive band at 264 nm and a negative band at 294 nm (Fig. 5). This CD inversion has been established as a characteristic feature of Z-conformation. In this study, native DNA showed a negative peak at around 244 nm and a positive peak (maxima) corresponding to 274 nm (Fig. 6a). After bromination in high salt, near inversion in the CD spectrum was noticed with a positive band at 255 nm and a negative band at 294 nm (Fig. 6b). For low salt brominated form, no spectral inversion was noticed.

UV Spectroscopic Behavior of DNA-Polyamine Complex

The UV spectroscopic behavior of native calf thymus DNA (50 µg/ml) complexed with 100 µg/ml each of spermine, spermidine and putrescine has been depicted in Table 4.

TABLE 3

UV Absorption Characteristcs of Native and Brominated DNA

Absorption Characteristics	DNA Polymers		
	native DNA	Br4	BrN
λ_{max} (nm)	258	258	258
λ_{min} (nm)	230	242	232
Absorbance at 260 nm	2.08	0.84	1.50
Percent decrease in absorbance at 260nm	0	59.6	27.5
Absorbance ratio (A_{295}/A_{260})	0.130	0.35	0.188

Br4 and BrN represent native calf thymus DNA brominated in 4 M and 0.15 M NaCl.

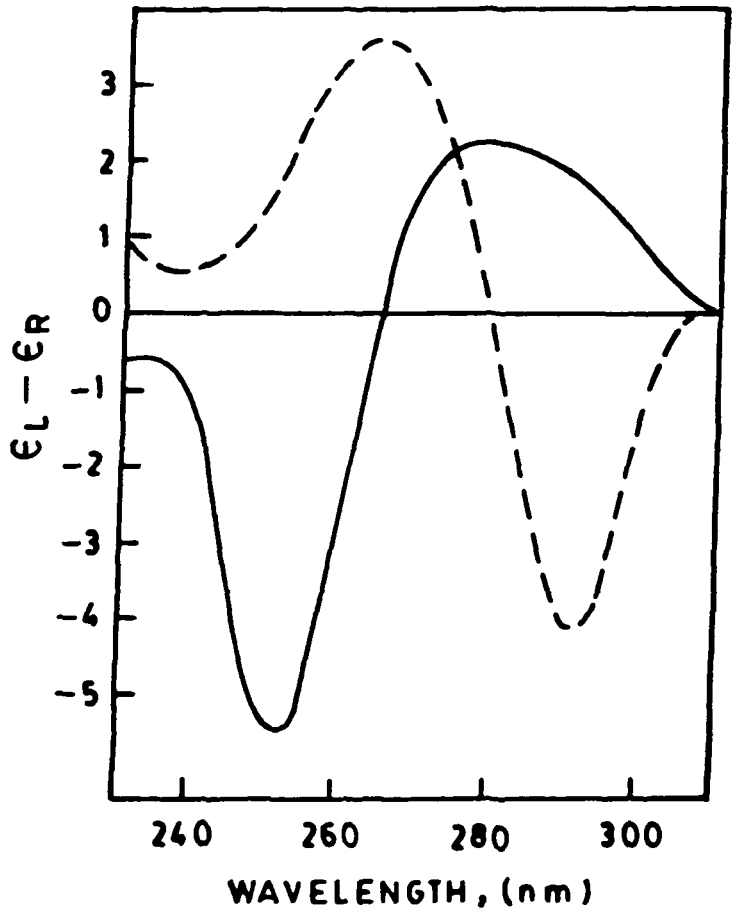


Fig.5. Circular dichroism spectra of poly(dG-dC).poly(dG-dC) in low (——) and high salt (-----) at pH 7.2. Taken from Pohl and Jovin, 1972.

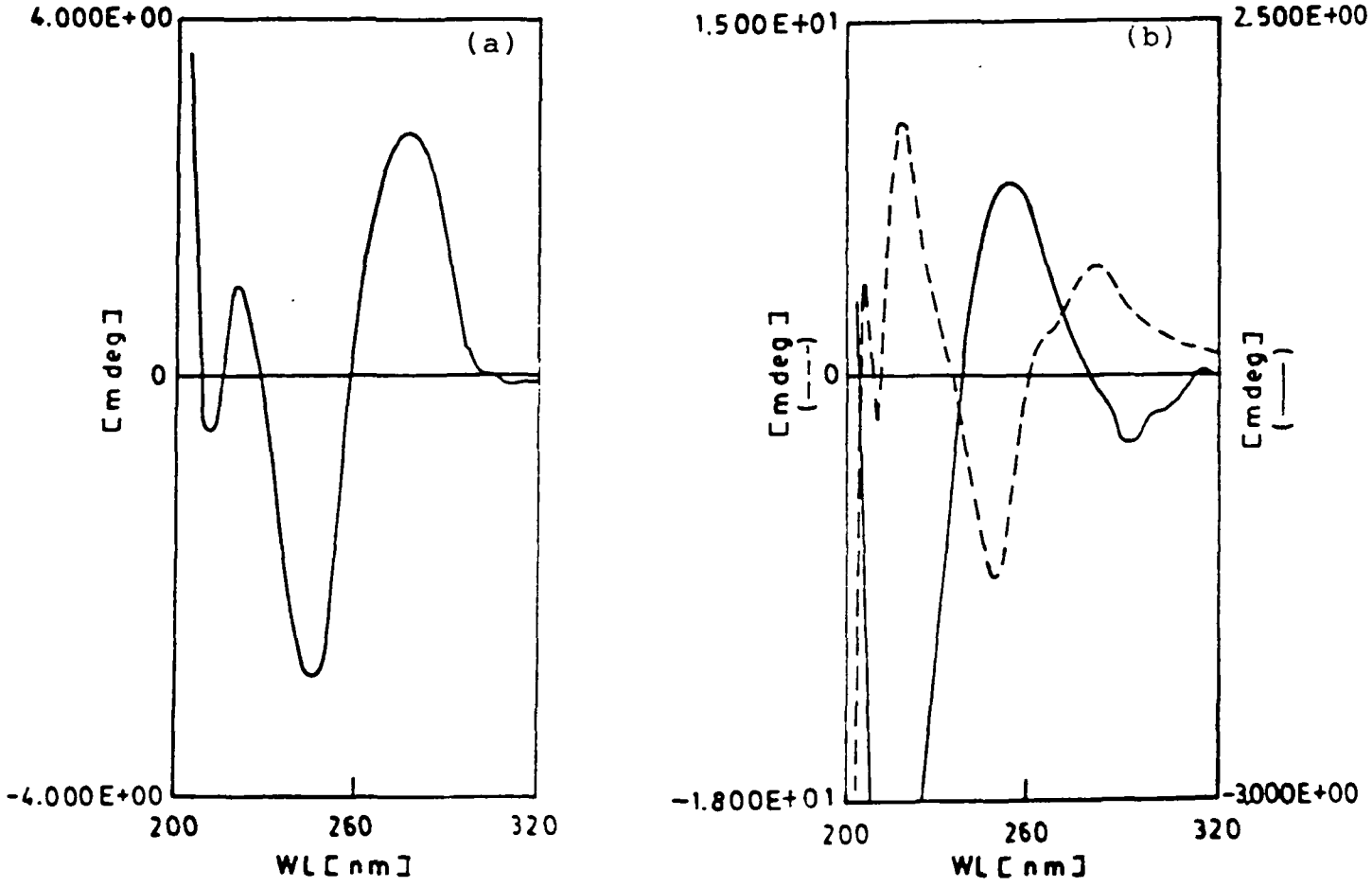


Fig.6. **(a)** Circular dichroism spectrum of native calf thymus DNA in TBS, pH 7.4.

(b) Circular dichroism spectra of native DNA brominated in 0.15M (----) and in 4.0M (——) NaCl. The spectra were recorded in TBS, pH 7.4.

TABLE 4

UV Absorption Characteristics of Native Calf Thymus DNA Complexed with Polyamines

native DNA complexed with	Absorbance at 260 nm	295 nm	Absorbance ratio (A_{295}/A_{260})	Percent relative increase in the absorbance ratio
--	1.473	0.153	0.104	---
Spermine	1.484	0.196	0.132	26.92
Spermidine	1.455	0.185	0.127	22.12
Putrescine	1.425	0.176	0.124	19.23

The data represent the arithmatic mean of triplicate samples. The absorbance of polyamine (100 ug/ml) alone was substracted from the sample containing native DNA and polyamines.

Native calf thymus DNA exhibited enhanced absorbance in the presence of polyamines. The data conclusively shows increased absorbance ratio in the presence of the above three polyamines. The percent relative increase in absorbance ratio was in the range of 19 to 27. On the other hand, when poly(dG-dC).poly(dG-dC) was exposed to varying concentrations of spermine, under identical conditions, it showed more than 60 percent increase in the absorbance ratio even when the polyamine concentration was 40 µg/ml (Table 5). Increased UV absorbance at around 295 nm has been found related to the structural perturbations in the classical B-DNA helix. It has earlier been shown that the double stranded poly(dG-dC) attains Z-conformation in 4 M NaCl and the observed absorbance ratio (A_{295}/A_{260}) of 0.30.

Binding of Anti-Z-DNA Antibodies to DNA-Polyamine Complex

The B→Z transition of purified native calf thymus DNA in the presence of polyamines was further probed by ELISA. The binding of anti Z-DNA antibodies to DNA in the presence of varying concentrations of spermine, spermidine and putrescine shows the B→Z transition of DNA. (Fig. 7). The enzyme immunoassay was conducted as described by Thomas and Messner (1988). The experimentally induced antibodies against Z-DNA did not recognize native DNA in the absence of polyamines. But, in their presence, the antibody binding was

TABLE 5

UV Absorption Characteristics of Poly (dG-dC).poly (dG-dC) Complexed with Varying Concentrations of Spermine

Poly(dG-dC) with Spermine (µg/ml)	Absorbance at 260 nm	295 nm	Absorbance ratio (A_{295}/A_{260})	Percent relative increase in the absorbance ratio
0	0.620	0.120	0.194	-
10	0.634	0.156	0.246	26.8
20	0.644	0.176	0.273	40.7
40	0.627	0.198	0.316	62.9
50	0.615	0.193	0.314	61.8
100	0.600	0.188	0.313	61.3

The data represent arithmatic mean of triplicate samples of control and experimental. The absorbance of spermine in each set was substracted from the experimental value.

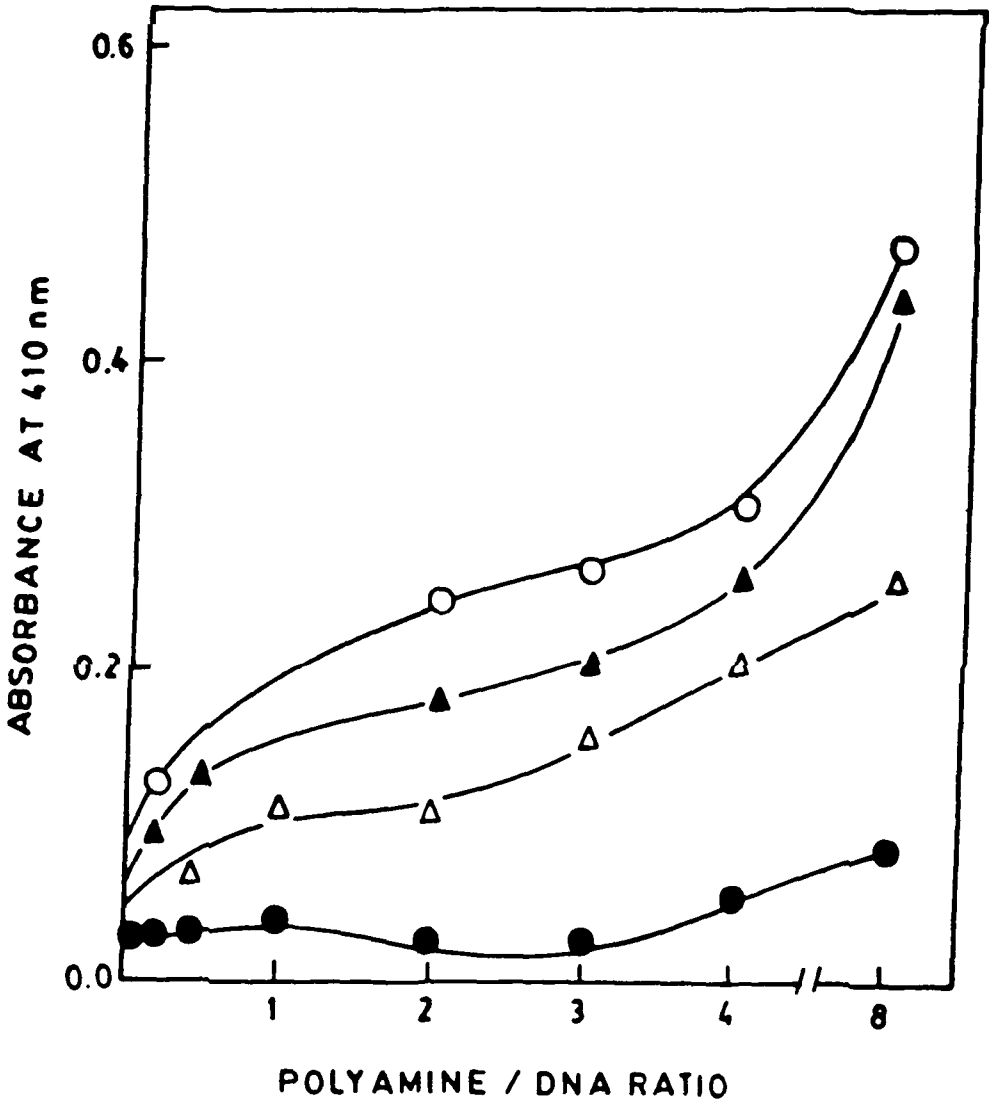


Fig.7. Enzyme-immunoassay detection of anti-Z-DNA antibody binding to native DNA (—●—), and native DNA complexed with varying amounts of spermine (—○—), spermidine(—▲—) and putrescine(—△—). The colour was developed for 45 min and absorbance read at 410 nm.

found to increase with increasing concentration of polyamines. In this case, both spermine and spermidine were almost equally effective in the conversion of B-DNA to Z-conformation while putrescine appears less effective. Double stranded poly(dG-dC) complexed with varying concentrations of polyamines served as the comparative control for the binding of anti-Z-DNA antibody to polyamine induced Z-conformation of DNA. Here, comparatively lesser amount of polyamines were required to bring about the said conformational change. Anti-Z-DNA antibodies showed no binding with double stranded poly (dG-dC) (Fig. 8).

Characterization of DNA-BSA and E_2-BSA-DNA Conjugates

Purified calf thymus DNA (average size 200 bp) was covalently linked to BSA and E_2-BSA using glutaraldehyde as the crosslinking reagent. Crosslinked product was purified free of unlinked species on a Sephadex G-200 column. The column fractions in both the cases were analysed for the presence of DNA and BSA by the method of Burton (1956)and Lowry et al. (1951) respectively (Figs. 9 & 10). These fractions were also monitored for absorbance at 260 nm and 280 nm. Fractions containing both DNA and protein were pooled and taken as DNA-BSA and E_2-BSA-DNA conjugates. These conjugates were analysed by ultraviolet spectroscopy (Table 6). A red shift of 8 nm (from 230 to 238 nm) and 11 nm (from 230 to 241 nm) was observed in the minima of DNA after its

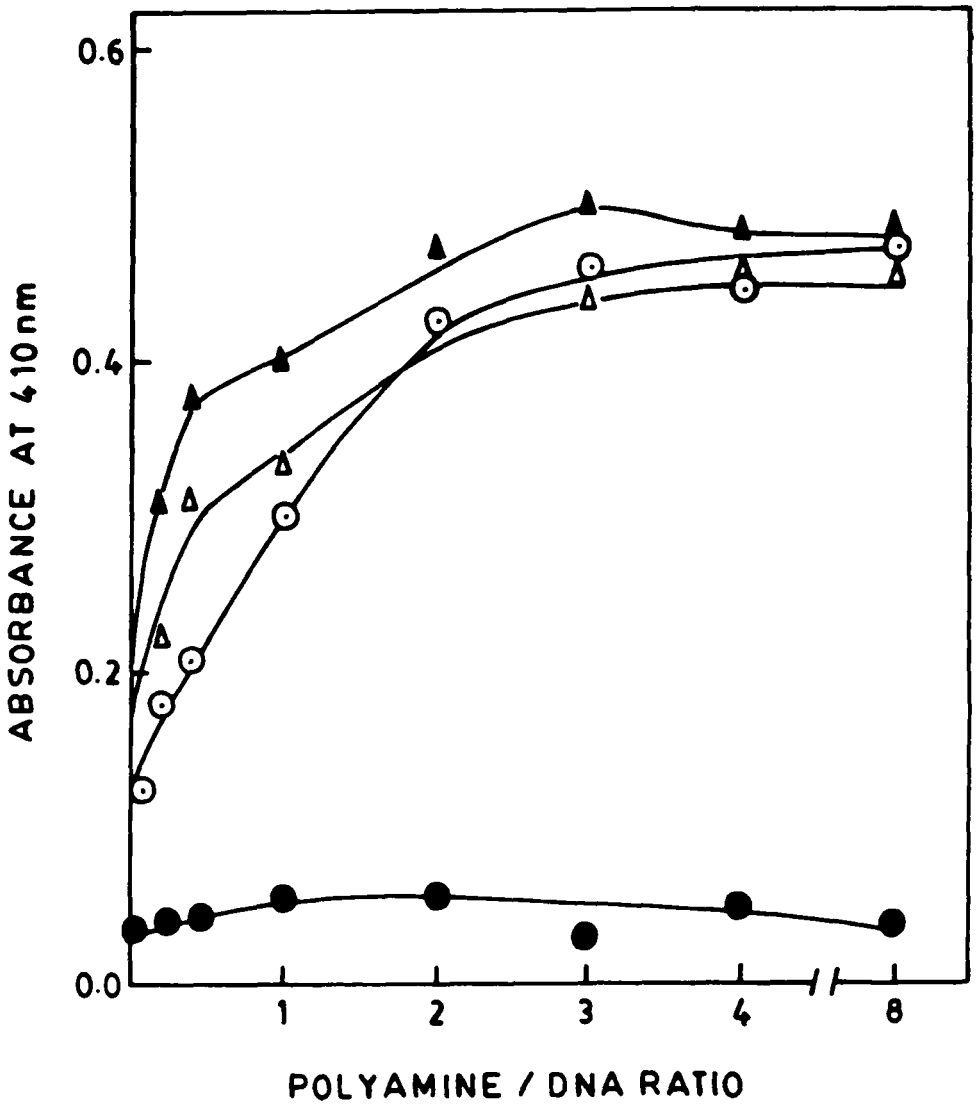


Fig.8. Enzyme-immunoassay titration of anti-Z-DNA antibody binding to poly(dG-dC).poly(dG-dC) (—●—), and the polymer mixed with varying amounts of spermine (—○—), spermidine (—▲—) and putrescine (—△—).

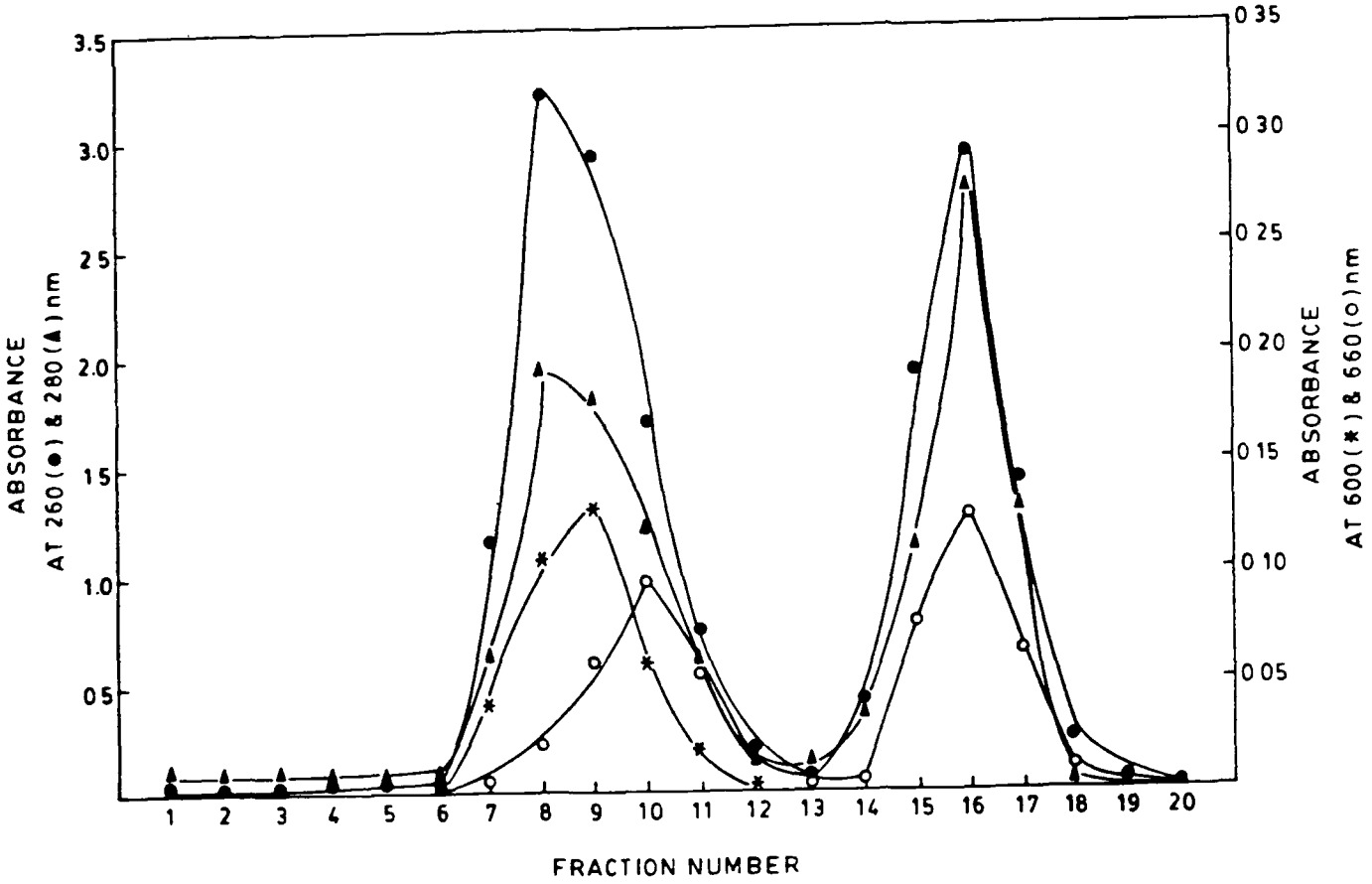


Fig.9. Elution profile of DNA-BSA conjugate on Sephadex G-200 column. Fractions of 2.5 ml were monitored for absorbance at 260 nm (—●—) and 280 nm (—▲—). Fractions were also analyzed for protein (—○—) and DNA (—*—) by the methods of Lowry et al. and Burton respectively.

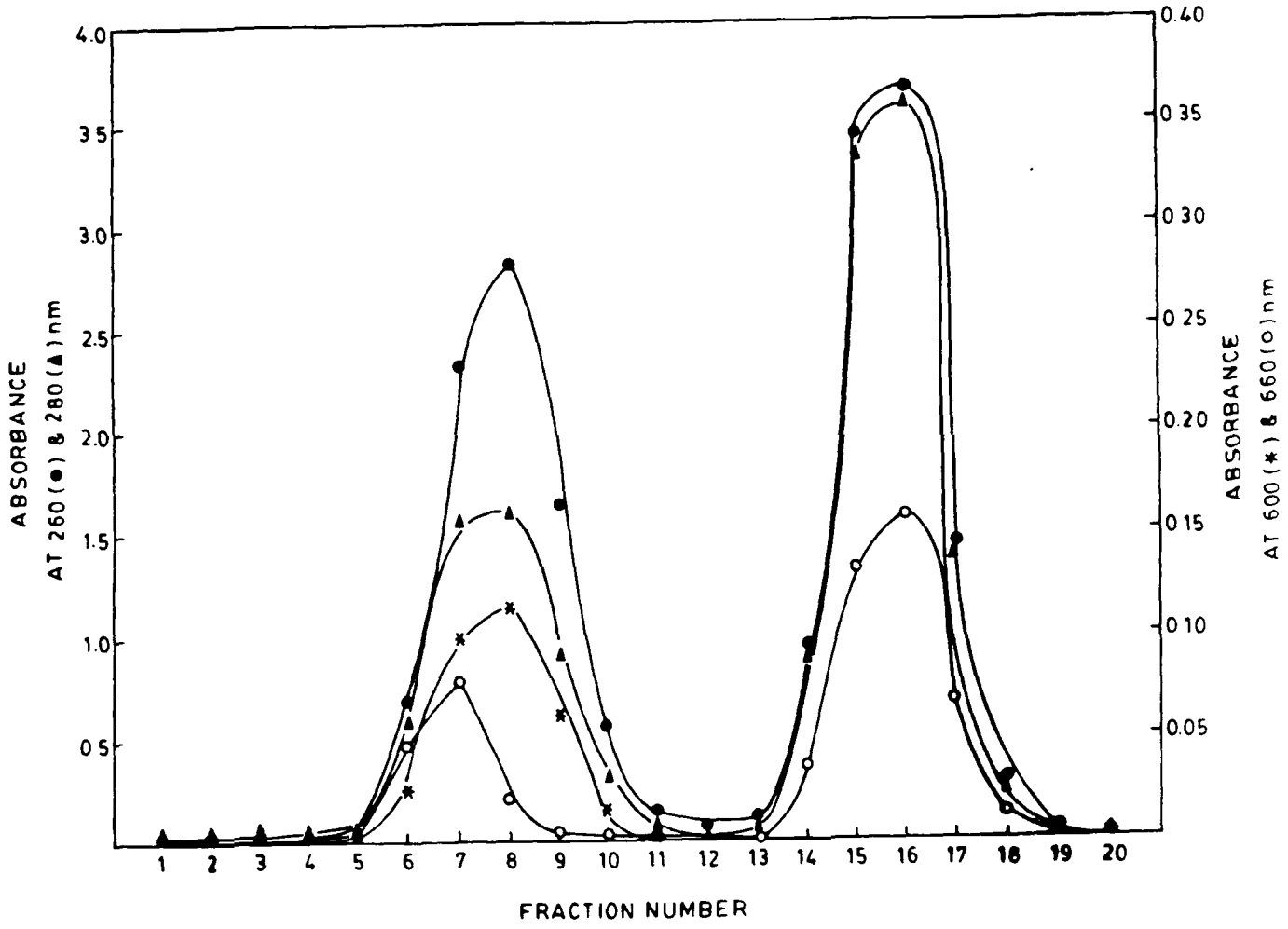


Fig.10. Elution profile of E_2-BSA-DNA conjugate on Sephadex G-200 column. Fractions of 2.5 ml were monitored for absorbance at 260 nm (—●—) and 280 nm (—▲—). Fractions were also analyzed for protein (—○—) and DNA (—*—) by the methods of Lowry et al. and Burton respectively.

TABLE 6

UV- Absorption Characteristics of Native and Modified DNA Fragment (average size 200 bp)

Species	λ_{max} (in nm)	λ_{min} (in nm)	A_{260}/A_{280}
DNA	258	230	2.2
BSA	278	250	0.6
DNA-BSA	258	238	1.6
E_2-BSA	278	255	0.88
E_2-BSA-DNA	258	241	1.45

linkage to BSA and E_2-BSA respectively (Figs. 11 & 12). The E_2-BSA and E_2-BSA-DNA spectra exhibited marked differences in their UV absorption pattern (Fig. 12). A blue shift of 14 nm in the minima and a shift of 20 nm in the maxima of E_2-BSA was observed after its covalent linkage to DNA. A substantial decrease in absorbance of linked E_2-BSA around 300 nm was also observed. Results of these studies indicate that DNA is linked to BSA and E_2-BSA.

Thermal Denaturation Studies

The thermal denaturation of native and modified DNA was investigated between 30°C and 95°C. The process was characterised by determining the percent fraction of nucleic acid in denatured state at various temperatures as well as by the evaluation of Tm. The increase in UV absorbance at 260 nm was recorded as a function of rise in temperature at a rate of 1.0°C/min and taken as a measure of the extent of denaturation.

When DNA-spermine complex was heated from 30°C to 95°C on an electronically controlled heating device, the helix opening started after 86°C and the melting temperature (Tm) was 89°C, while in the case of corresponding control (i.e. native DNA), significant heat induced strand separation was noticed after 85°C and Tm was 87°C (Fig. 13).

While in another set of experiments, when native DNA

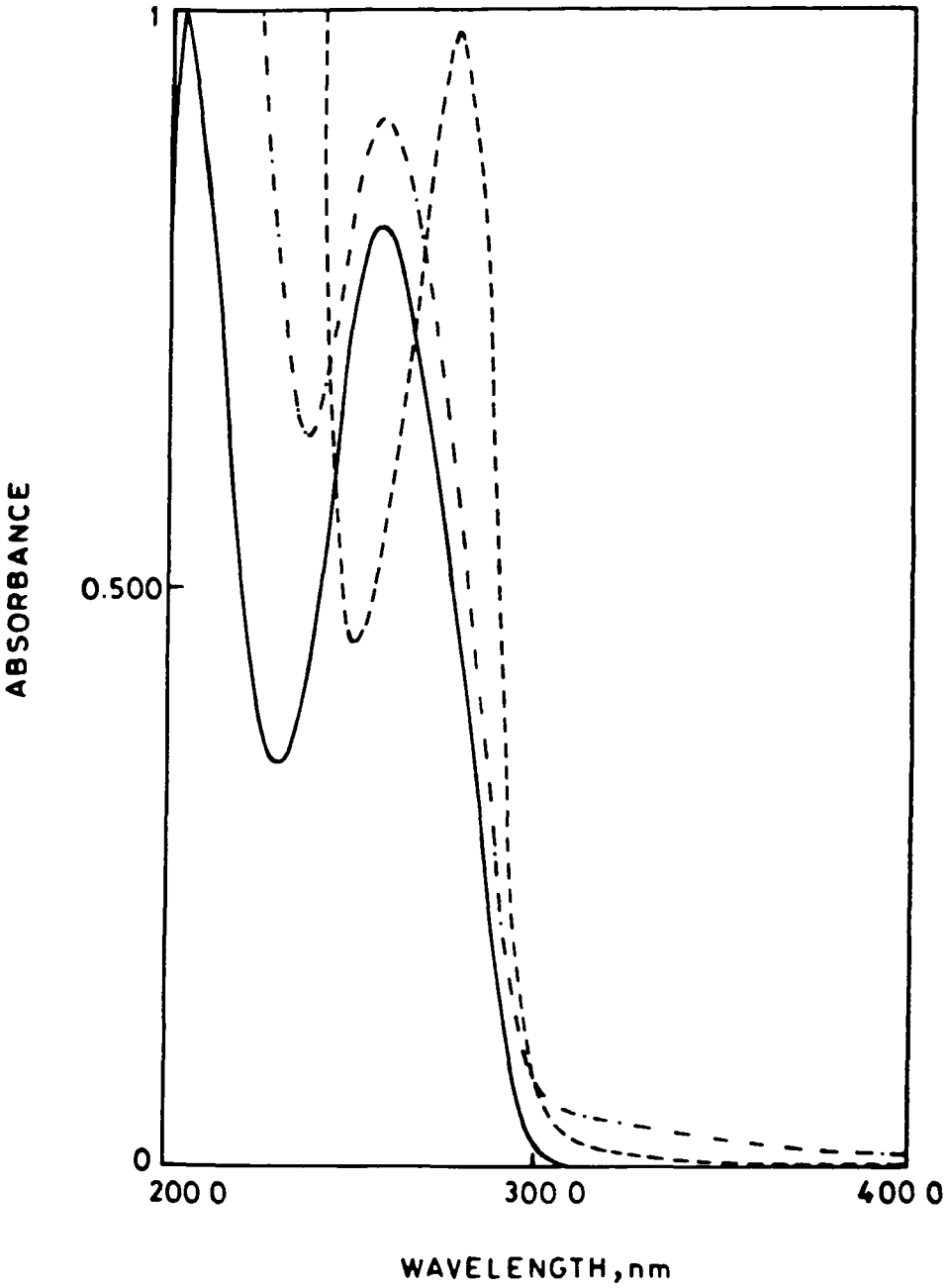


Fig.11. Ultraviolet absorption spectra of DNA (——), BSA (- - - -) and DNA-BSA conjugate (-·-·-).

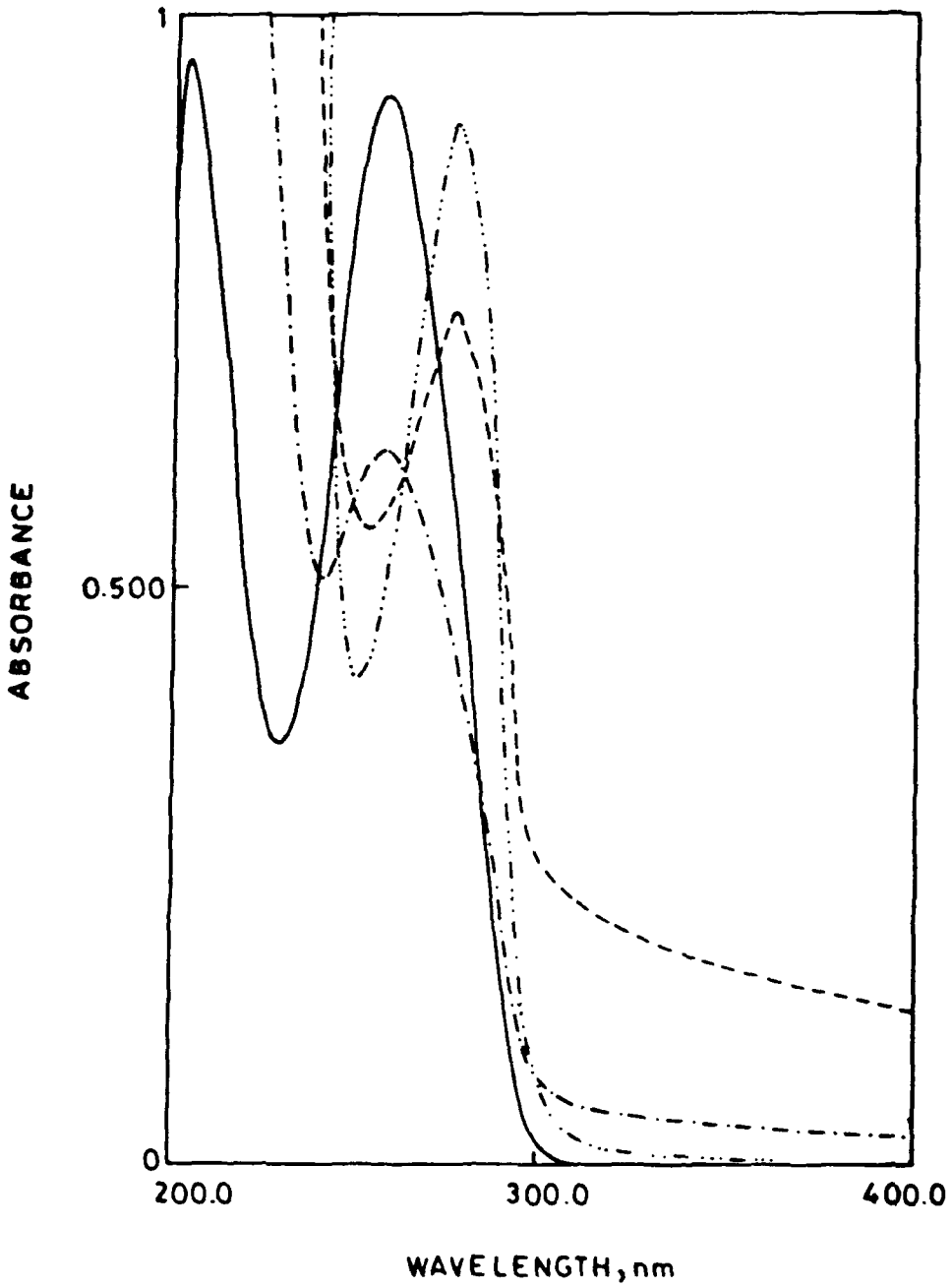


Fig.12. Ultraviolet absorption spectra of DNA (——), BSA (—·—·—), E_2-BSA (- - - -) and E_2-BSA-DNA conjugate (—·—·—).

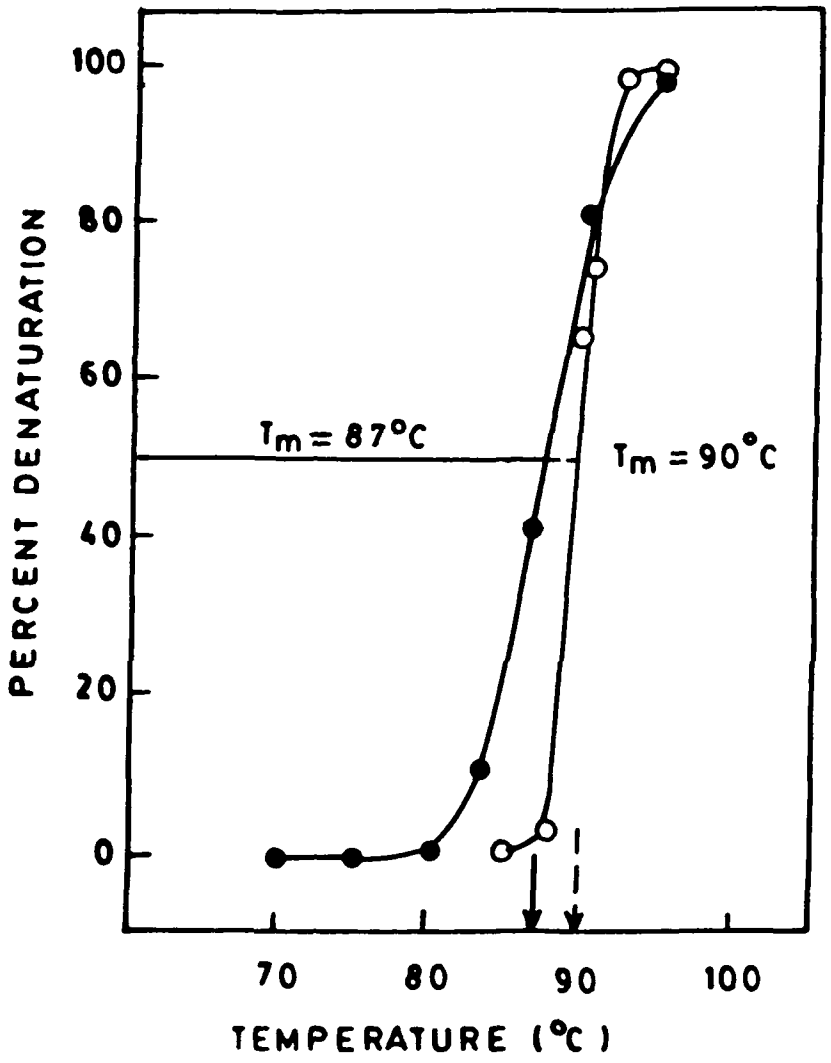


Fig.13. Melting profile of native DNA (—●—) and DNA-spermine complex (—○—). Melting temperatures were recorded at an increment of 1°C/min.

(~200 bp) and its modified forms, viz., E_2-BSA-DNA and DNA-BSA conjugates were subjected to heat induced helix opening, no denaturation or strand separation was visualized till 75°C in case of DNA and E_2-BSA-DNA conjugate. While in case of DNA-BSA conjugate, the duplex melting started at 50°C. The Tm values for native DNA (~200 bp), E_2-BSA-DNA and DNA-BSA were 83.5°C, 85°C and 82°C respectively (Fig. 14).

Thermodynamical Analysis of Unmodified and Modified Nucleic Acids

The modifications incurred in native DNA and native DNA fragments of around 200 base pairs as a result of spermine, BSA and E_2-BSA interactions was also investigated by means of thermodynamic analysis (Table 7). The free energy of thermal denaturation (ΔG_D) for native DNA was found to decrease linearly from 30°C-85°C. The ΔG_D for native DNA was computed to be -14.85 K.cal.deg^{-1} at 40°C and -6.6507 K.cal.deg^{-1} at 85°C. The high negative values for ΔG_D from 30°C-85°C indicate the tremendous stability exhibited by the double helical nucleic acid. Moreover, the large negative values till 85°C indicate that the thermal energy being implied on the native DNA to break the bonds between guanine and cytosine (G≡C) and adenine and thymine (A=T) was encountering a strong opposition from these stable bonds and thus the DNA double helix was not appreciably disrupted till 85°C. On the other hand, further elevation in temperature

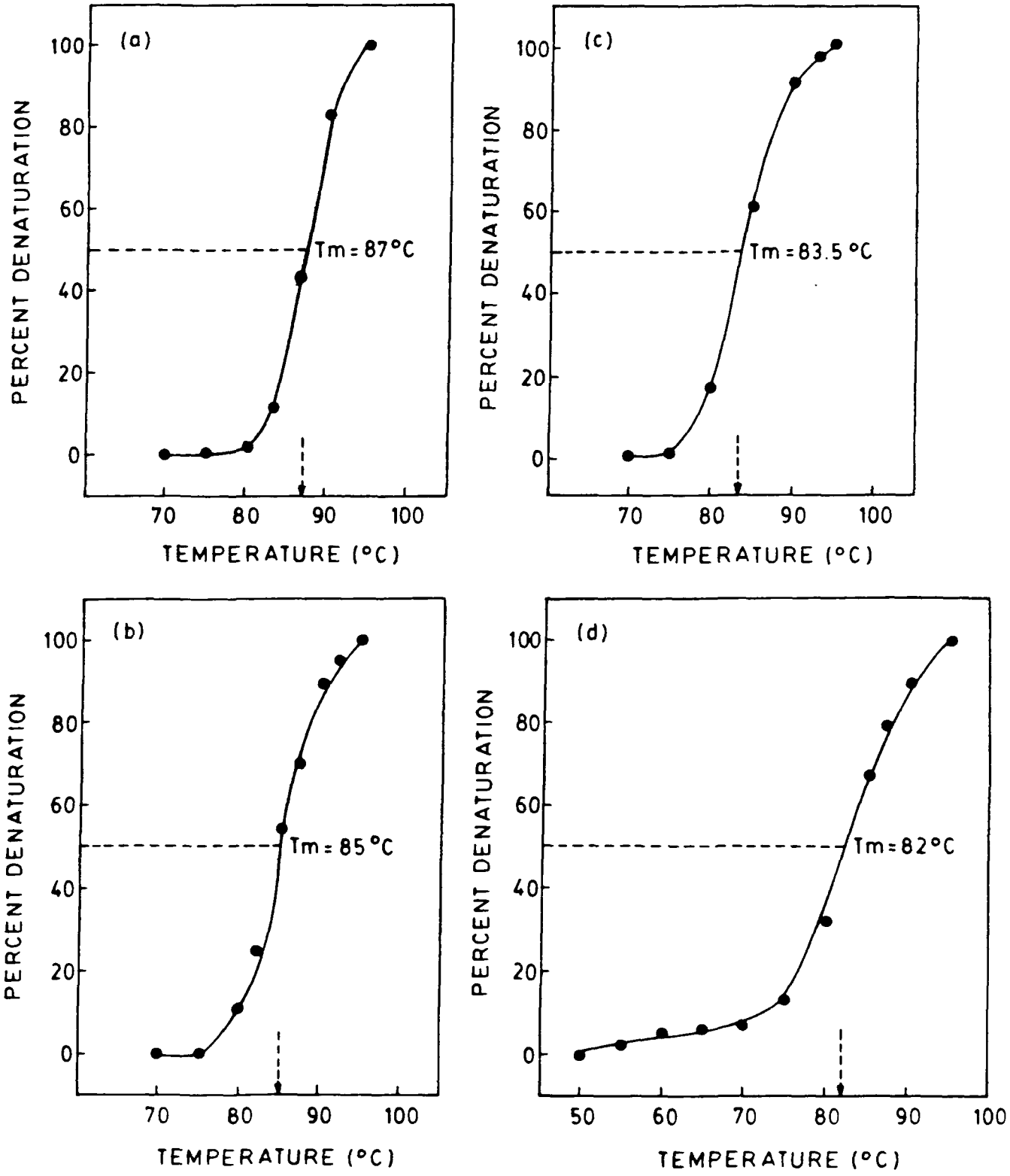


Fig.14. Thermal denaturation curves of native DNA (a), E_2-BSA-DNA conjugate (b), native DNA [~200bp] (c) and DNA-BSA conjugate (d)

TABLE 7

Thermodynamic Characteristics of Unmodified/Modified Nucleic Acids

Temperature (Abs.)	ΔG_D in K.cal.deg.$^{-1}$				
	native DNA	native DNA (~200 bp)	DNA-Spermine	DNA-BSA	E_2-BSA-DNA
308.15	-	-12.531	-8.146	-	-9.234
313.15	-14.850	-10.951	-6.575	-12.551	-7.104
318.15	-11.450	-11.126	-5.713	-12.751	-6.395
323.15	-10.158	-13.141	-4.623	12.907	-5.886
328.15	-9.126	-	-3.970	10.064	-5.726
333.15	-8.262	-13.548	-3.514	7.776	-5.475
338.15	-8.073	-	-3.808	7.496	-5.557
343.15	-8.192	-10.863	-3.406	7.245	-5.749
348.15	-8.164	14.112	-2.813	5.500	-6.490
350.15	-	14.193	-	-	-6.221
353.15	-8.058	4.889	-2.616	2.143	5.881
355.15	-	-	-	-	3.216
358.15	-6.650	-1.187	-0.467	2.173	-0.542
359.65	11.160	-	-0.407	-	-
360.15	-	-2.711	-	-3.942	-
361.15	4.623	-	2.751	-	2.534
363.15	0.281	-6.684	-1.897	-7.051	-6.254
364.15	-0.687	-	-3.153	-	-
365.15	-3.961	-	-	-7.045	-
366.15	-	-10.550	-	-	-9.031
367.15	-16.586	-	-	-	-

(from 86.5°C to 90°C) showed positive ΔG_D values. The ΔG_D values were computed to be 11.16 K.cal.deg^{-1} at 86.5°C and 0.281 K.cal.deg^{-1} at 90°C. The positive values of ΔG_D here indicate the transition from native to the denatured state. Further elevation in temperature showed a shift in ΔG_D values from the positive scale towards the negative side, thereby indicating the complete attainment of separation of the two strands as a result of thermal denaturation.

In the case of DNA-spermine complex, the ΔG_D was computed to be -8.146 K.cal.deg^{-1} at 35°C which decreased linearly to -0.407 at 86.5°C. The large negative ΔG_D values from the initial stages of melting till 86.5°C indicates that the interaction of spermine with DNA resulted in the formation of a more stable nucleic acid conformer, judged on comparative basis of ΔG_D values, which was found to decrease linearly till 86.5°C in DNA-spermine complex. Thus, the ΔG_D values depicted in Table 7 indicate DNA-spermine complex to be more thermodynamically stable than native DNA.

In contrast to native DNA, the ΔG_D values for 200 bp DNA fragment decreased linearly from 35°C to 70°C and not till 85°C as was the case with native DNA. Comparison of the ΔG_D values indicate that the 200 bp DNA fragment was thermodynamically less stable than native DNA.

The thermodynamical analysis of 200 bp DNA linked to BSA via glutaraldehyde (DNA-BSA) exhibited negative ΔG_D

values only till 45°C. The ΔG_D value at 45°C was computed to be -12.751 K.cal.deg^{-1}. Further elevation in temperature exhibited a sharp transition of the ΔG_D values from the negative side towards the positive scale. The abrupt shift in the negative ΔG_D values above 45°C to positive ΔG_D value (+12.907 K.cal. deg^{-1} at 50°C) indicates that some portions of the DNA-BSA complex were already in the unstacked form. The results depicted in Table 7, reveal that DNA-BSA complex was thermodynamically less stable than 200 bp DNA.

In case of E_2-BSA-DNA, the ΔG_D values decreased linearly from 35°C to 77°C. The ΔG_D value was computed out to -9.234 K.cal.deg^{-1} at 35°C and -6.221 K.cal.deg^{-1} at 77°C. However, further elevation in temperature resulted in the shift of ΔG_D values towards positive side. Furthermore, comparative analysis with 200 bp DNA shows that the E_2-BSA-DNA macromolecule exhibited negative ΔG_D values right from the initial stages of melting till 77°C whereas, 200 bp DNA exhibited negative ΔG_D values till 70°C. The negative ΔG_D values before melting signify the degree of stability of the macromolecule. Thus, the results indicate that E_2-BSA-DNA was thermodynamically more stable than DNA of 200 bp.

Detection of Modification by Time Course Kinetic Analysis

The process of modification or/perturbations in the

native DNA as well as 200 bp native DNA fragment as a result of spermine, BSA and E_2-BSA interactions was characterized by the measurements of fractions of nucleic acid in the denatured state, f_D, with time, 't'.

As evident from Fig. 15, the f_D values for native DNA were of every low magnitude upto 55 min of heating (85°C). However, an abrupt increase in f_D value was observed from 56 to 64 min corresponding to thermal denaturation temperatures of 86°C to 94°C. The $t_{1/2}$ (half-life) of thermal denaturation for native DNA was computed to be 59 min. On the contrary, DNA-spermine complex formed as a result of ionic interaction exhibited a very low magnitude of f_D values upto 58 min of heating (88°C). Thus, in comparison to native DNA, the DNA-spermine complex showed very low magnitude of f_D values for a greater period of heating time (i.e. by 3 min). The half life ($t_{1/2}$) for thermal denaturation of DNA-spermine complex was computed to be 60 min. The results indicate that DNA-spermine complex was thermodynamically more stable than native DNA.

In case of 200 bp DNA, no progressive increase in f_D values was observed till 45 min (75°C) (Fig 15). A rapid increase in the f_D value was observed from 45-65 min of heating at (75°C-93°C). The $t_{1/2}$ value in this case was found to be 55 min (85°C).

In contrast to 200 bp DNA, the DNA (200 bp) linked

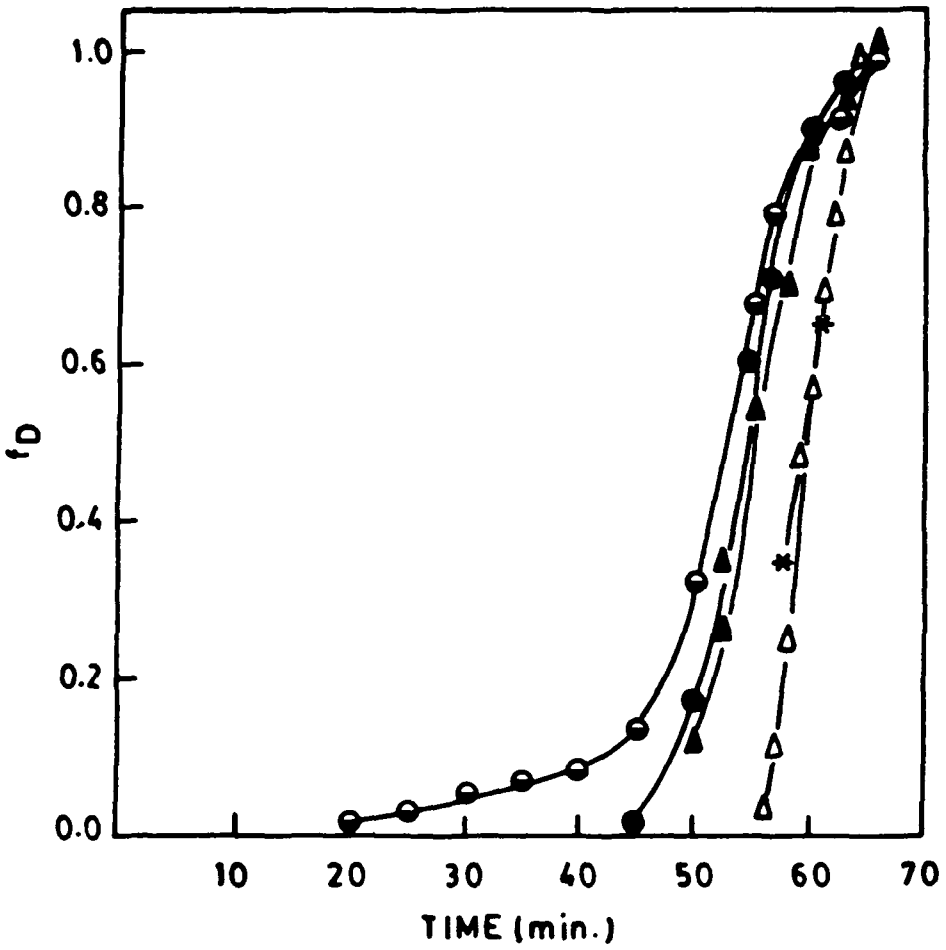


Fig.15. Time course kinetic plot for thermal transition of modified and unmodified nucleic acids. Native DNA (—△—), DNA-spermine complex (—*—), ~200 bp DNA (—●—), DNA-BSA (—◒—) and E_2-BSA-DNA (—▲—).

covalently to BSA via glutaraldehyde exhibited a low f_D value only till 20 min of heating (50°C) (Fig. 15). However, further elevation in temperature till 60 min of heating produced an abrupt increase in its f_D values. The $t_{1/2}$ here was computed to be 51.5 min. The results show that DNA-BSA started melting 25 minutes earlier than native 200 bp DNA, thereby indicating that covalent conjugation of BSA via glutaraldehyde to 200 bp DNA resulted in the unstacking of the DNA macromolecule, thereby rendering it thermodynamically less stable.

However, when E_2-BSA-DNA was subjected to thermal-kinetic studies, it showed an f_D value of low magnitude till 50 min of heating (80°C) (Fig. 15). Comparative analysis with 200 bp DNA shows that E_2-BSA-DNA required an additional 5 min of heating as revealed by the f_D values, thereby indicating that coupling of E_2-BSA to 200 bp DNA resulted in the formation of a thermodynamically more stable conformer. The half life ($t_{1/2}$) in this case was computed out to be 56 min.

Also, the order of the denaturation process was determined by plotting $-\ln(Y_{fD(\infty)}-Y_{fD(t)})$ versus time. A straight line for the modified and unmodified DNA indicates the first order rate denaturation. The apparent first order rate constant was determined from the slope of the plot. Native DNA (200 bp), DNA-spermine complex, E_2-BSA-DNA and

DNA-BSA exhibited apparent first order rate constants of the order of 4.5045×10^{-4} sec^{-1}, 5.316×10^{-3} sec^{-1}, 2.1645×10^{-4} sec^{-1} and 2.4038×10^{-4} sec^{-1} respectively (Figs. 16 to 19). These kinetic parameters are depicted in Table 8.

Production and Characterization of Antibodies Against E_2-BSA, E_2-BSA-DNA and DNA-BSA Conjugates

Antibodies against E_2-BSA, E_2-BSA-DNA and DNA-BSA conjugates were induced in female rabbits. The serum was separated from the immunized blood samples and tested for the presence of antibodies, against their respective immunogens, by immunodiffusion, counterimmunoelectrophoresis and ELISA techniques.

(a) Antibodies against E_2-BSA conjugate

Anti-E_2-BSA antibodies were found to be non-precipitating as indicated by immunodiffusion. The antibody titer was found to be >1:12800 (Fig. 20) in direct binding ELISA. Preimmune serum showed low binding. The specificity of these antibodies was checked by competition ELISA using immunizing antigen as the inhibitor. The maximum inhibition in the antibody binding was 93 percent at an inhibitor concentration of 15 µg/ml (Fig. 21). Fifty percent inhibition was obtained at an inhibitor concentration of 1.0 µg ml^{-1}. Anti-E_2-BSA antibody binding was subjected to competition from various inhibitors. Besides the immunizing

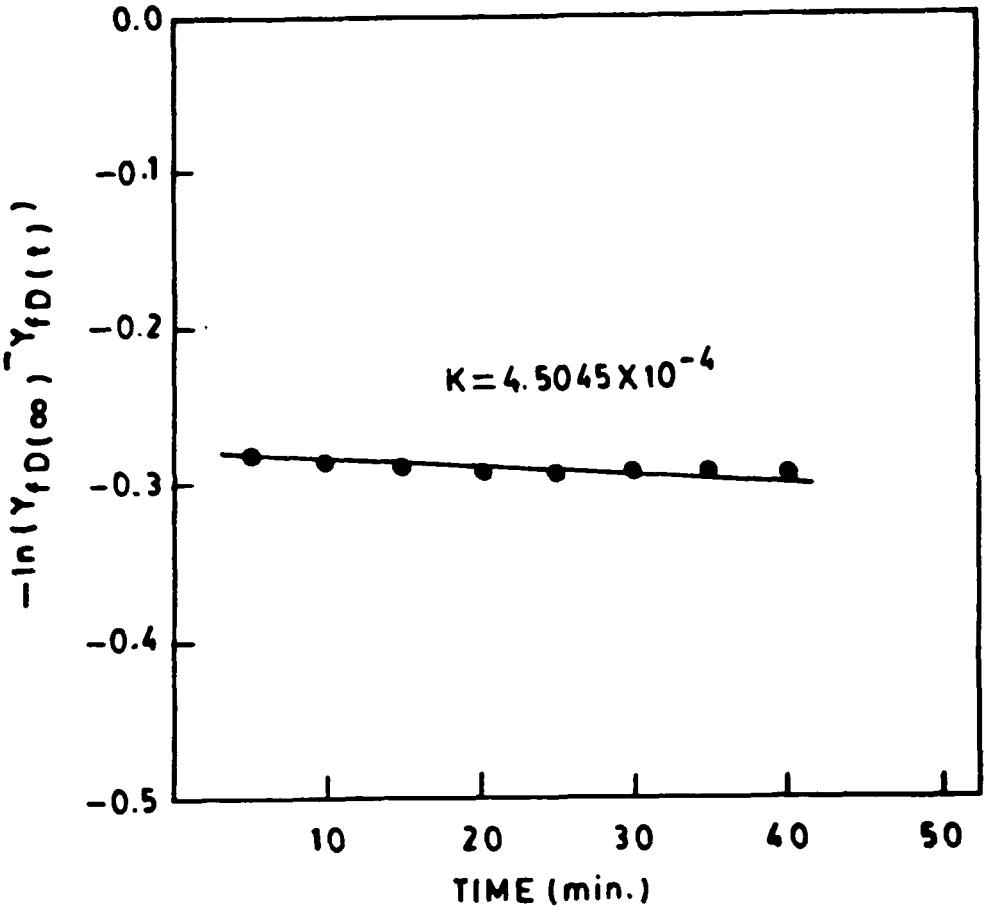


Fig.16. First order thermal denaturation profile of native DNA (~200 bp).

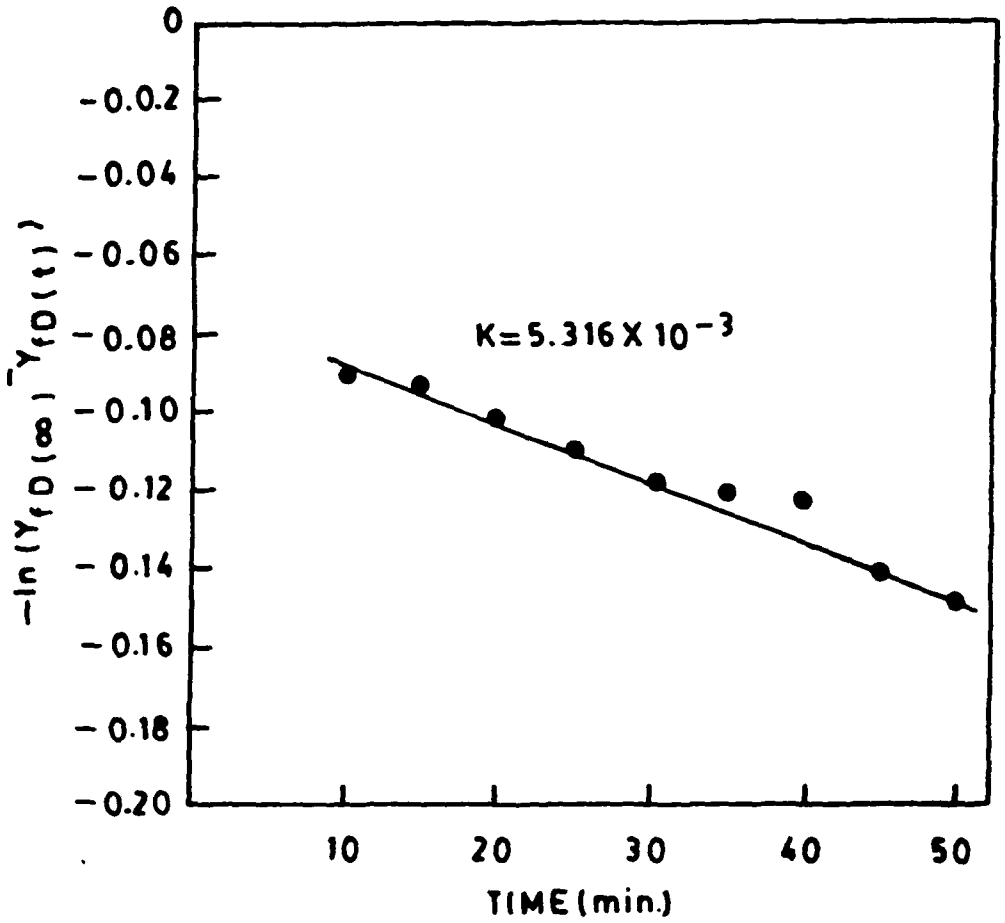


Fig.17. First order thermal denaturation profile of DNA-spermine complex.

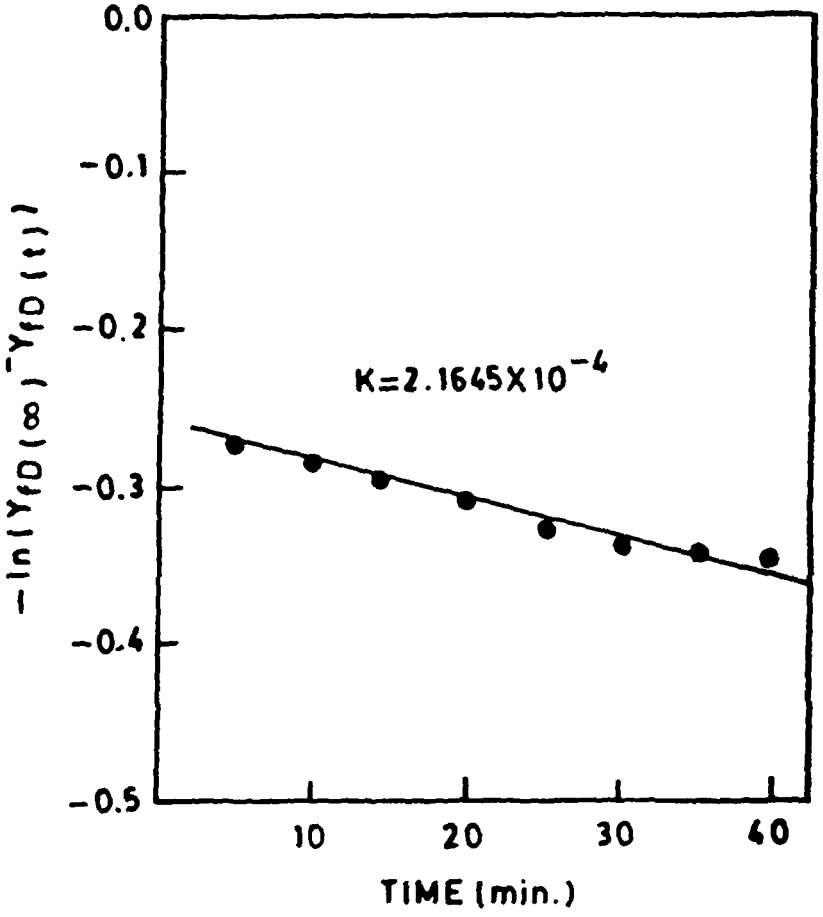


Fig.18. First order thermal denaturation profile of E_2-BSA-DNA conjugate.

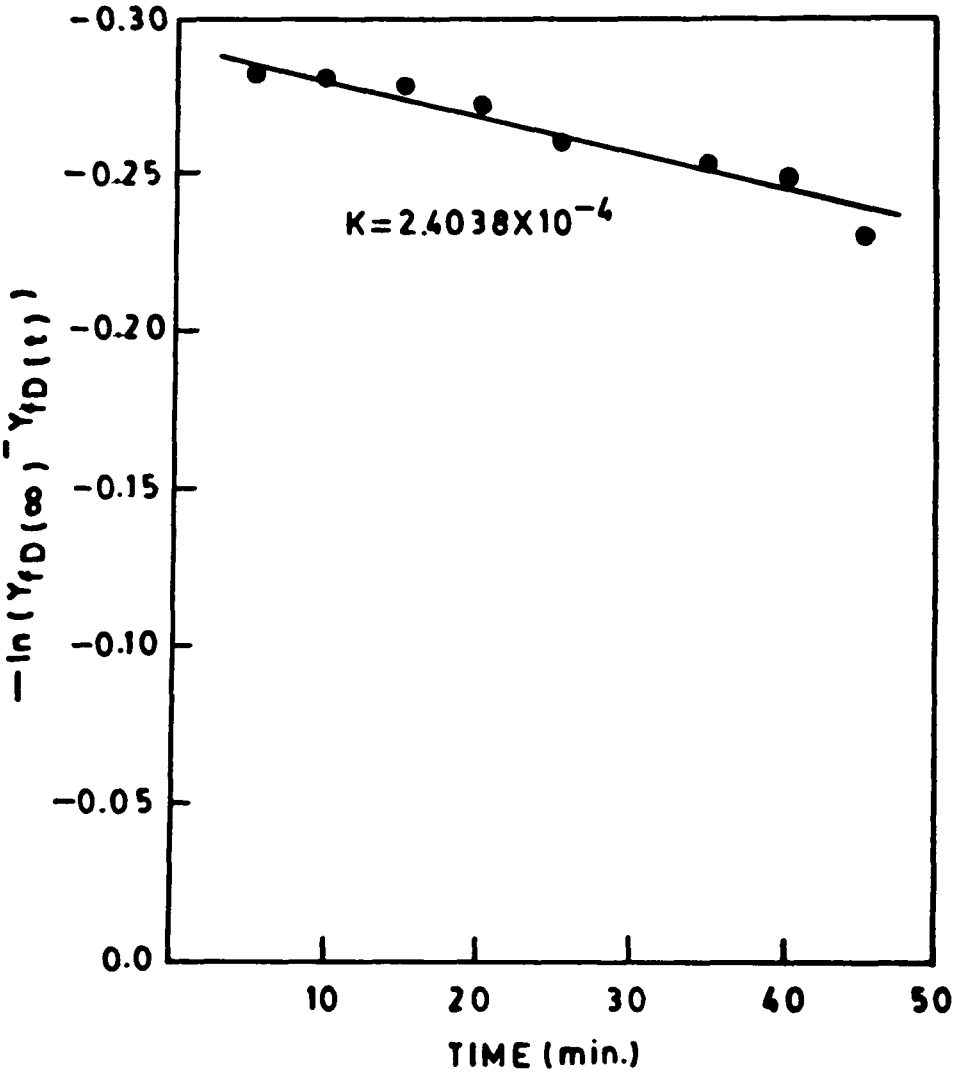


Fig.19. First order thermal denaturation profile of DNA-BSA conjugate.

TABLE 8

Apparent First Order Rate Constants for Thermal Denaturation

Nucleic Acid	App.First Order Rate Constant (K)
1. native DNA-Spermine	5.316×10^{-3} sec^{-1}
2. native DNA (~200 bp)	4.5045×10^{-4} sec^{-1}
3. E_2-BSA-DNA	2.1645×10^{-4} sec^{-1}
4. DNA-BSA	2.4038×10^{-4} sec^{-1}

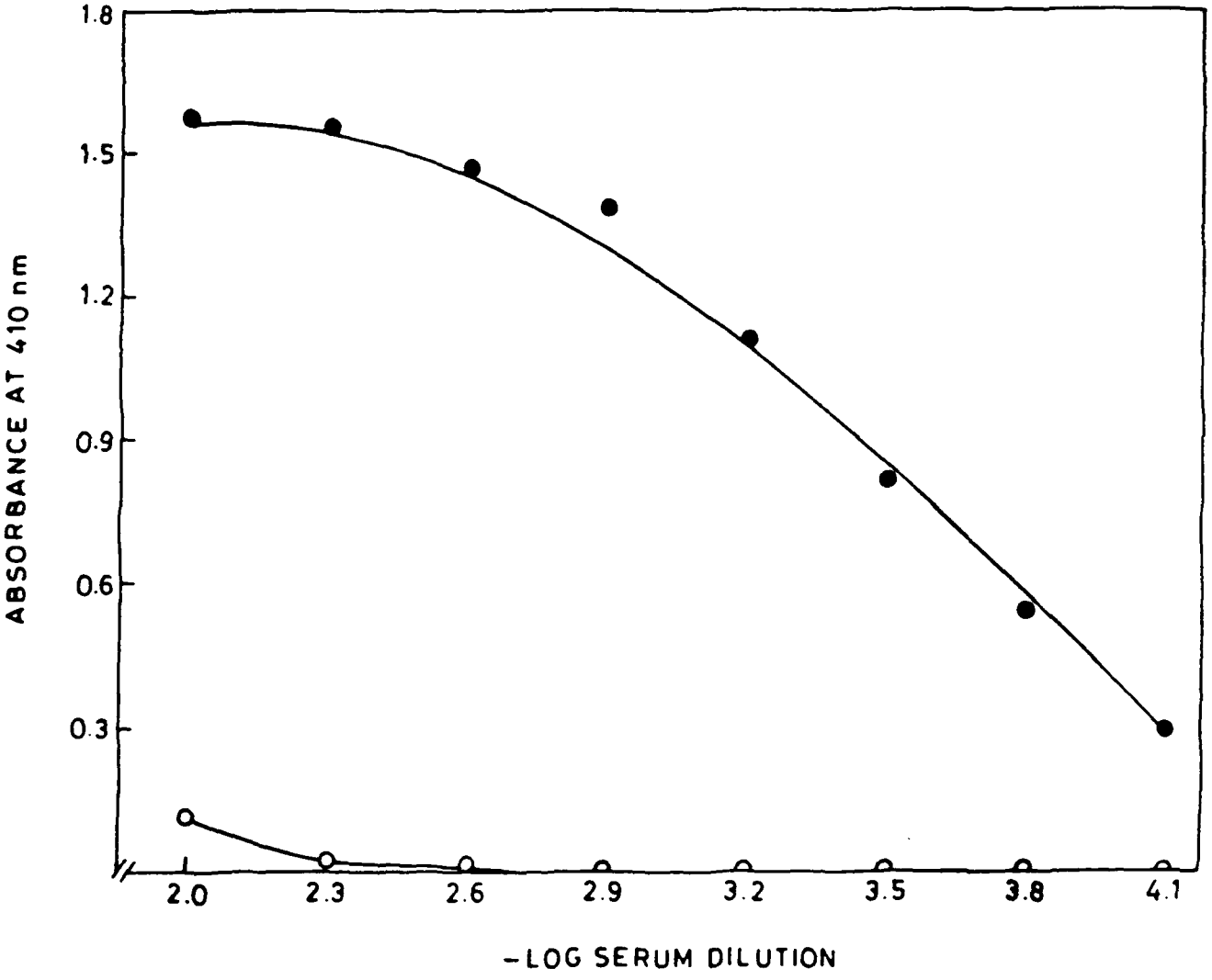


Fig.20. Direct binding ELISA of anti-E_2-BSA antibodies. The plate was coated with E_2-BSA. Immunized (—●—) and preimmune (—○—) serum.

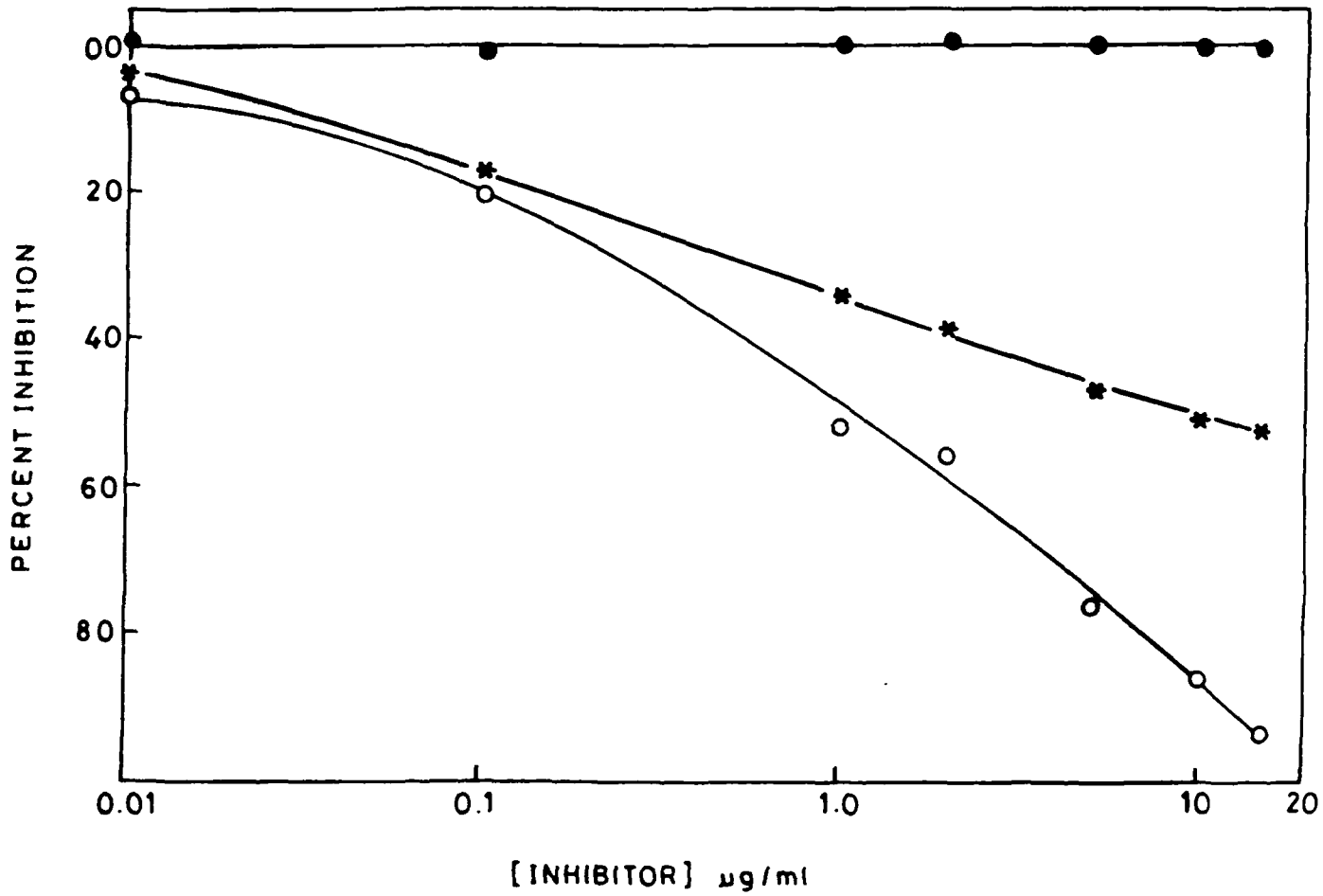


Fig.21. Inhibition ELISA of anti-E_2-BSA antibodies. The competitors were ß-estradiol (—∗—), E_2-BSA (—○—) and BSA (—●—).

antigen, 86%, 53% and 23% inhibitions were obtained with E_2-BSA-DNA, estradiol and histone (H_2A) respectively at an inhibitor concentration of 15 µg ml^{-1}. Inhibition with native DNA, ssDNA, RNA, Br-DNA, poly(rG). poly(dC), DNA-BSA and BSA was negligible (Table 9; Figs. 22 & 23).

(b) Antibodies against E_2-BSA-DNA conjugate

Antibodies raised against E_2-BSA-DNA conjugate were also non-precipitating. The antibody titer as determined by direct binding ELISA was >1:12800 (Fig. 24). The binding of preimmune serum was of low magnitude. The specificity of induced antibodies was explored by competition assay. Table 10 shows the results obtained with various inhibitors. Studies were also performed on ion-exchange and affinity purified IgG. A maximum of 91% inhibition in the antibody (IgG) activity was caused when immunogen was used as inhibitor (Fig. 25). Fifty percent inhibition was observed at an inhibitor concentration of 0.09 µg ml^{-1}. Besides the immunogen, significant inhibition was caused by estradiol-albumin and ß-estradiol (Fig. 25). Almost negligible inhibition was obtained with native DNA, DNA-BSA, poly(rG).poly(dC), Br-DNA and histone. Heat denatured DNA and RNA were inhibitory but at a higher inhibitor concentration (Figs. 25 & 26; Table 11). Results obtained with affinity purified anti-E_2-BSA-DNA IgG are shown in Table 12.

TABLE 9

Competitive Inhibition of Anti-E_2-BSA Antibodies

Competitor	Concentration for 50% inhibition (µg/ml)	Percentage inhibition	Percentage relative affinity
E_2-BSA	1.0	93.0	100
E_2-BSA-DNA	1.2	86.0	83
DNA-BSA	-	-	-
ß-estradiol	9.0	53.0	11
BSA	-	-	-
Poly(rG).poly(dC)	-	-	-
Histone (H_2A	-	23.0	-
native DNA	-	-	-
ss DNA	-	-	-
RNA	-	-	-
Br-DNA	-	-	-

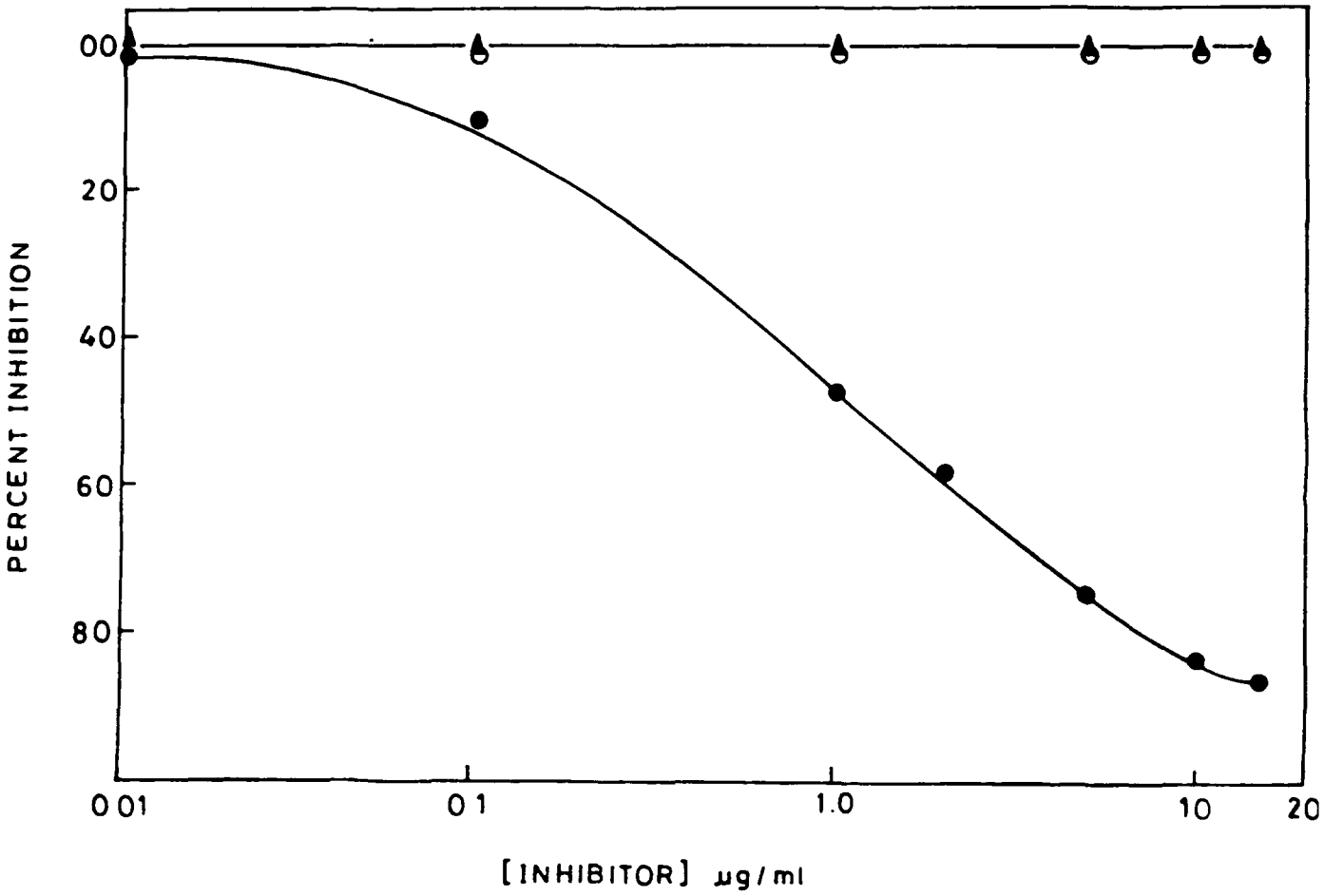


Fig.22. Inhibition of anti-E_2-BSA antibody activity by E_2-BSA-DNA(—●—), DNA-BSA(—⊖—) and native DNA(—▲—).

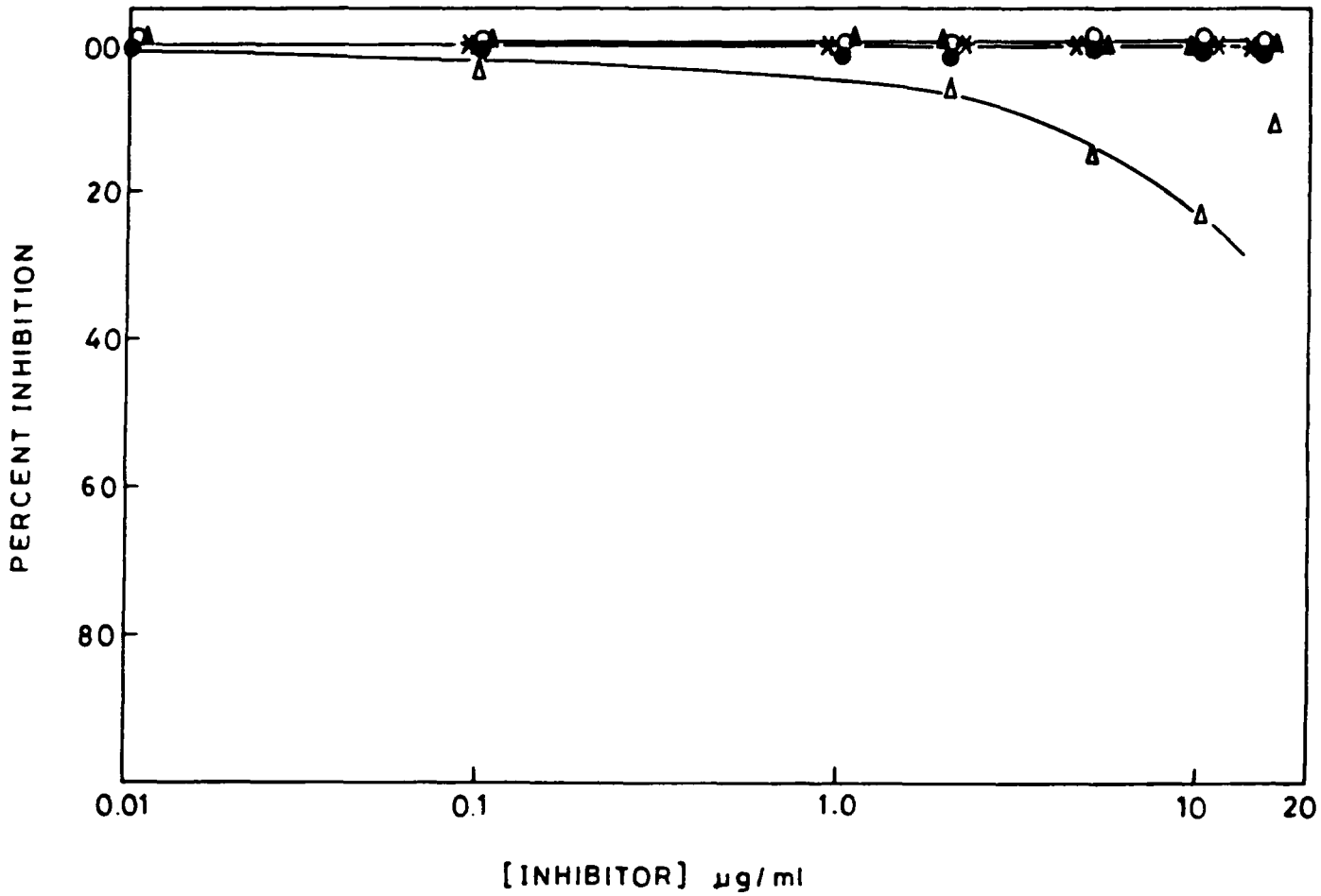


Fig.23. Inhibition of anti-E_2-BSA antibody activity by RNA (—●—), ssDNA (—*—), poly(rG).poly(dC) (—▲—), Br-DNA (—○—) and histone [H_2A] (—△—).

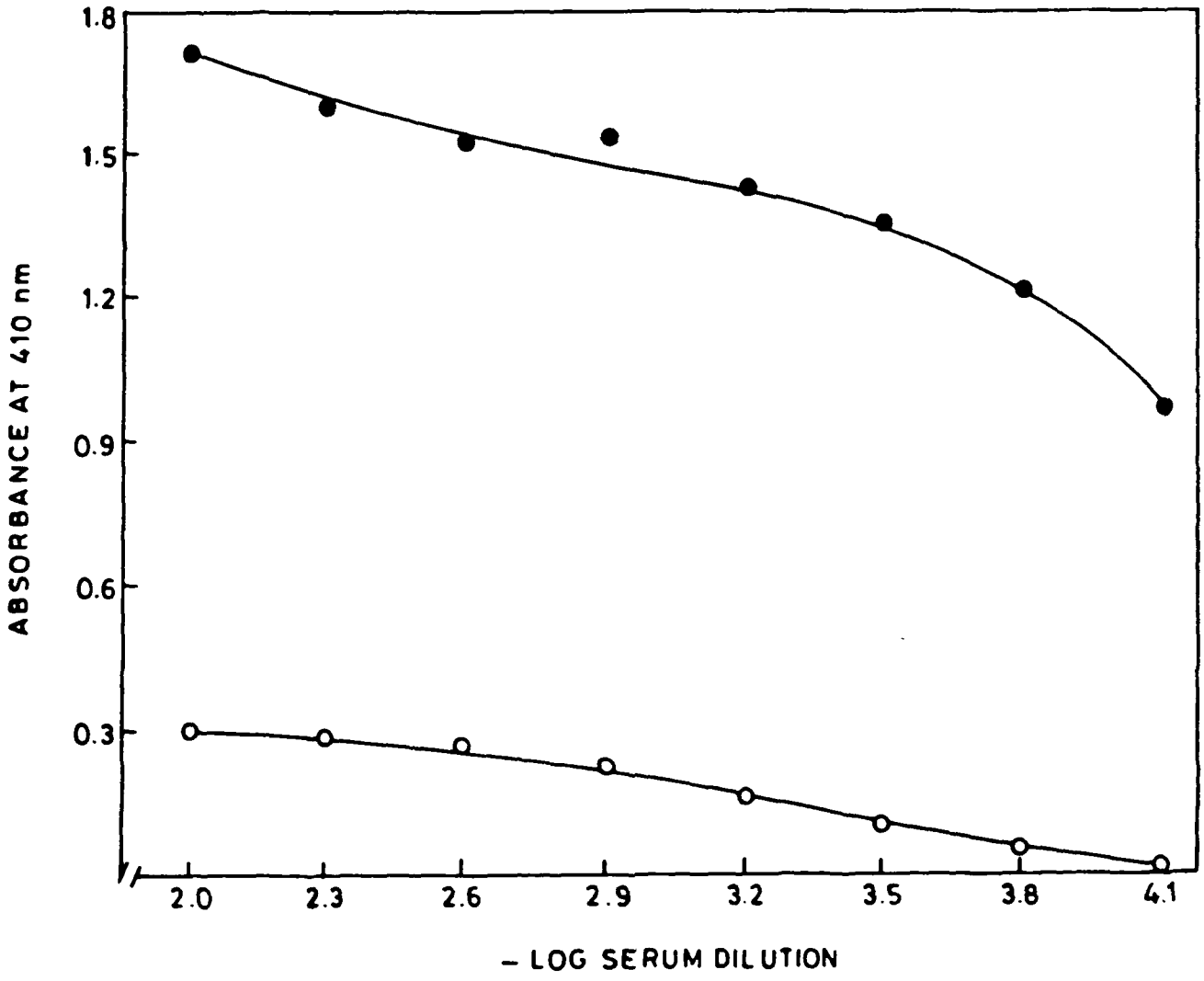


Fig.24. Direct binding ELISA of anti-E_2-BSA-DNA antibodies on the plate coated with the immunogen. Immunized (—●—) and preimmune (—○—) serum.

TABLE 10

Competitive Inhibition Data of Anti-E_2-BSA-DNA Antibodies

Competitor	Concentration for 50% inhibition (µg/ml)	Percentage inhibition
E_2-BSA-DNA	1.2	81
E_2-BSA	1.0	80
ß-estradiol	-	48
native DNA	-	-
DNA-BSA	-	-
BSA	-	-
Heat denatured DNA	-	38
RNA	-	31
Brominated DNA	-	-
Poly(rG).poly(dC)	-	-
Histone (H_2A)	-	18.0

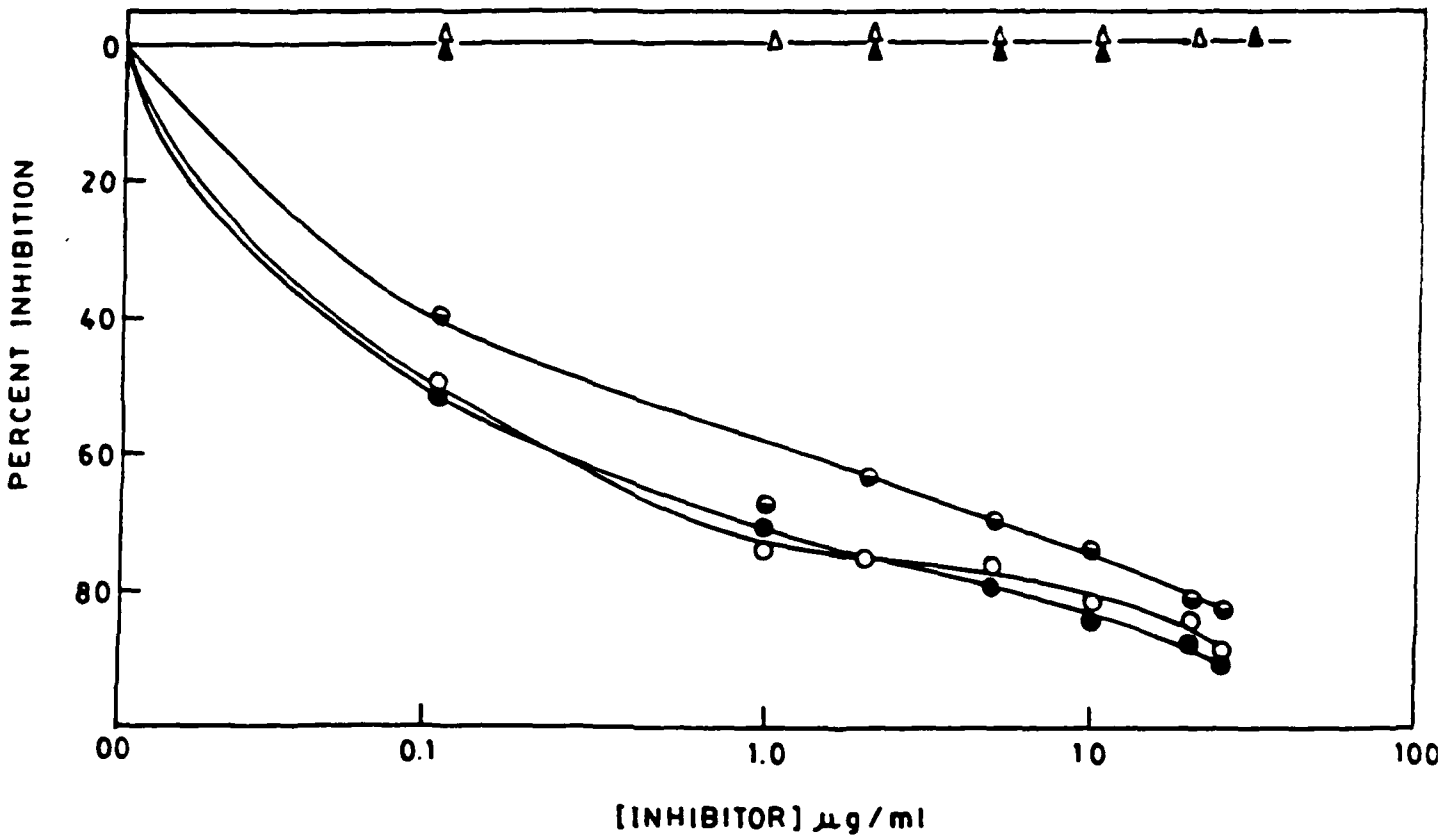


Fig.25. Inhibition of anti-E_2-BSA-DNA IgG by E_2-BSA (—○—), E_2-BSA-DNA (—●—), DNA-BSA (—△—), ß-estradiol (—◒—) and Br-DNA (—▲—).

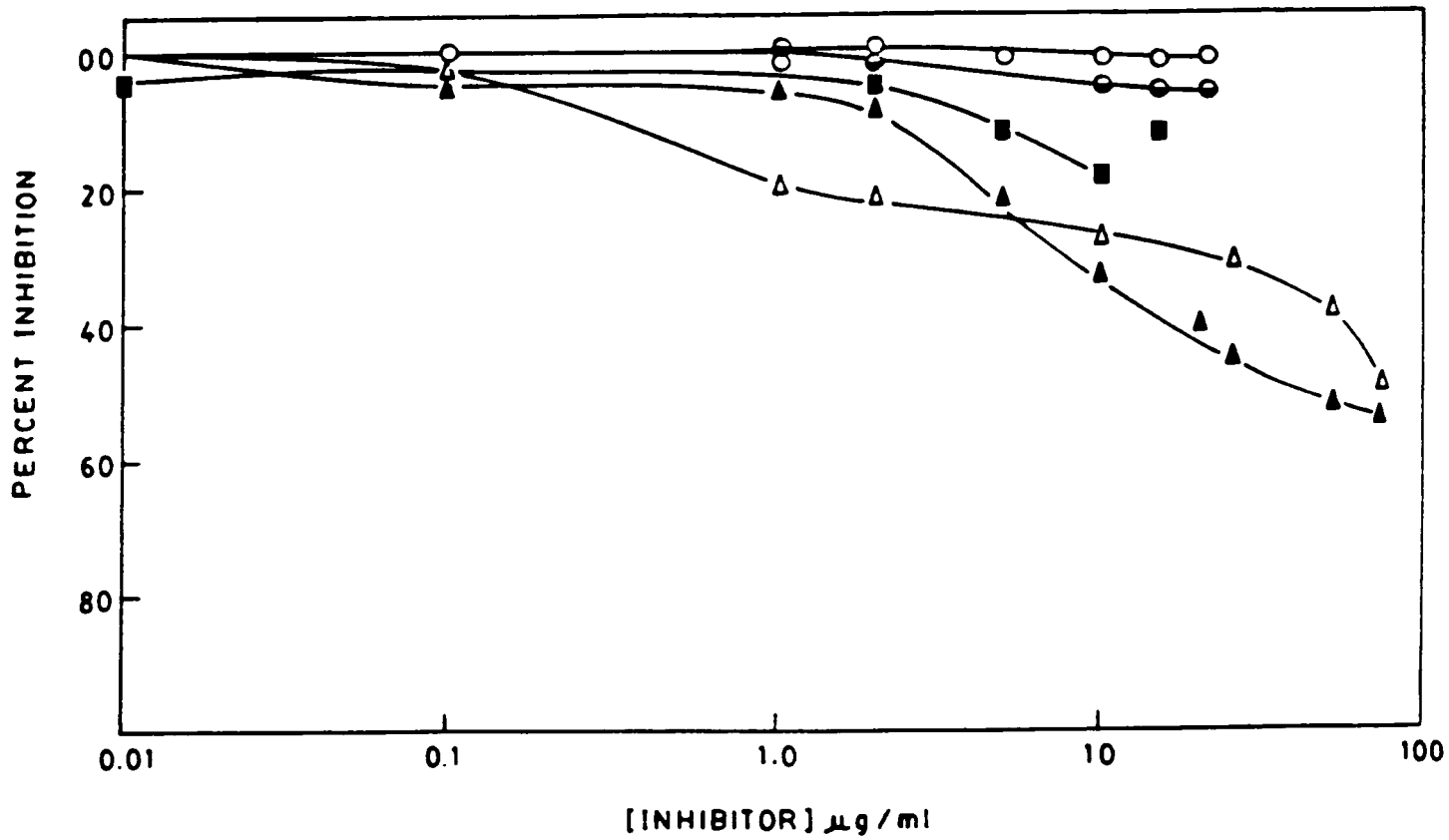


Fig.26. Inhibition of anti-E_2-BSA-DNA IgG. The competitors were native DNA (—○—), ssDNA (—▲—) RNA (—△—), poly(rG).poly(dC) (—◒—) histone [H_2A] (—■—).

TABLE 11

Competitive Inhibition Data of Anti-E_2-BSA-DNA IgG

Competitor	Concentration for 50% inhibition (µg/ml)	Percentage inhibition	Percent relative affinity
E_2-BSA-DNA	0.09	91	100
E_2-BSA	0.1	89	90
ß-estradiol	0.3	83	30
native DNA	-	-	-
DNA-BSA	-	-	-
BSA	-	-	-
Heat denatured DNA	40.0	54*	0.2
RNA	-	49*	-
Brominated DNA	-	-	-
poly(rG). poly(dC)	-	-	-
Histone(H_2A)	-	19	-

* inhibition at 75 µg/ml.

TABLE 12

Inhibition of Affinity Purified Anti-E_2-BSA-DNA IgG

Competitor	Concentration for 50% inhibition (µg/ml)	Percentage inhibition	Percentage relative affinity
E_2-BSA-DNA	0.08	95	100
E_2-BSA	0.1	87	80
ß-estradiol	0.4	80	20
native DNA	-	-	-
DNA-BSA	-	-	-
BSA	-	-	-
Heat denatured DNA	36	57*	0.22
RNA	60	51*	0.13
Brominated DNA	-	-	-
Poly(rG). poly(dC)	-	-	-

* inhibition at 75 µg/ml

(c) Antibodies against DNA-BSA conjugate

Anti-DNA-BSA antibodies were found to be precipitating as a sharp precipitin line was evident in immunodiffusion (Fig. 27). Direct binding ELISA on polystyrene plates coated with the immunizing antigen indicated high binding activity (>1:1600) (Fig. 28). To check the antibody specificity, competition ELISA was performed by employing DNA-BSA as inhibitor. A maximum of 83% inhibition in the antibody activity was recorded at an inhibitor concentration of 15 μg ml^{-1} (Fig. 29). Fifty percent inhibition was seen at an effective inhibitor concentration of 1.4 μg ml^{-1}. Varying degrees of inhibition in the antibody activity was obtained with various inhibitors. Native DNA, ss DNA, RNA, E_2-BSA, E_2-BSA-DNA, poly(rG).poly(dC) and Br-DNA were not inhibitory (Figs. 29 & 30).

Recognition of Brominated DNA by SLE Autoantibodies.

The interaction of anti-DNA autoantibodies with the Z-conformation of poly (dG-dC).poly(dG-dC) has been reported (Lafer et al., 1983b; Bergen et al., 1987; Krishna et al., 1993). Very few studies (Ali et al. 1991; Ali and Ali., 1993) have reported B→Z conversion in calf thymus DNA and its subsequent recognition by anti-DNA autoantibodies. The binding by SLE autoantibodies to native DNA brominated in high salt was checked in direct binding ELISA. The results

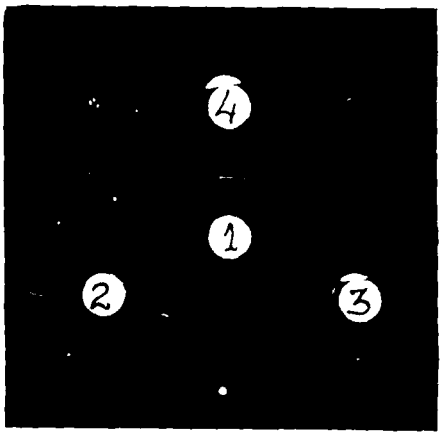


Fig.27. Immuno-crossreactivity of anti-DNA-BSA antibodies.
1. DNA-BSA conjugate.
2. Anti-E_2-BSA-DNA antibody.
3. Anti-E_2-BSA antibody.
4. Anti-DNA-BSA antibody.

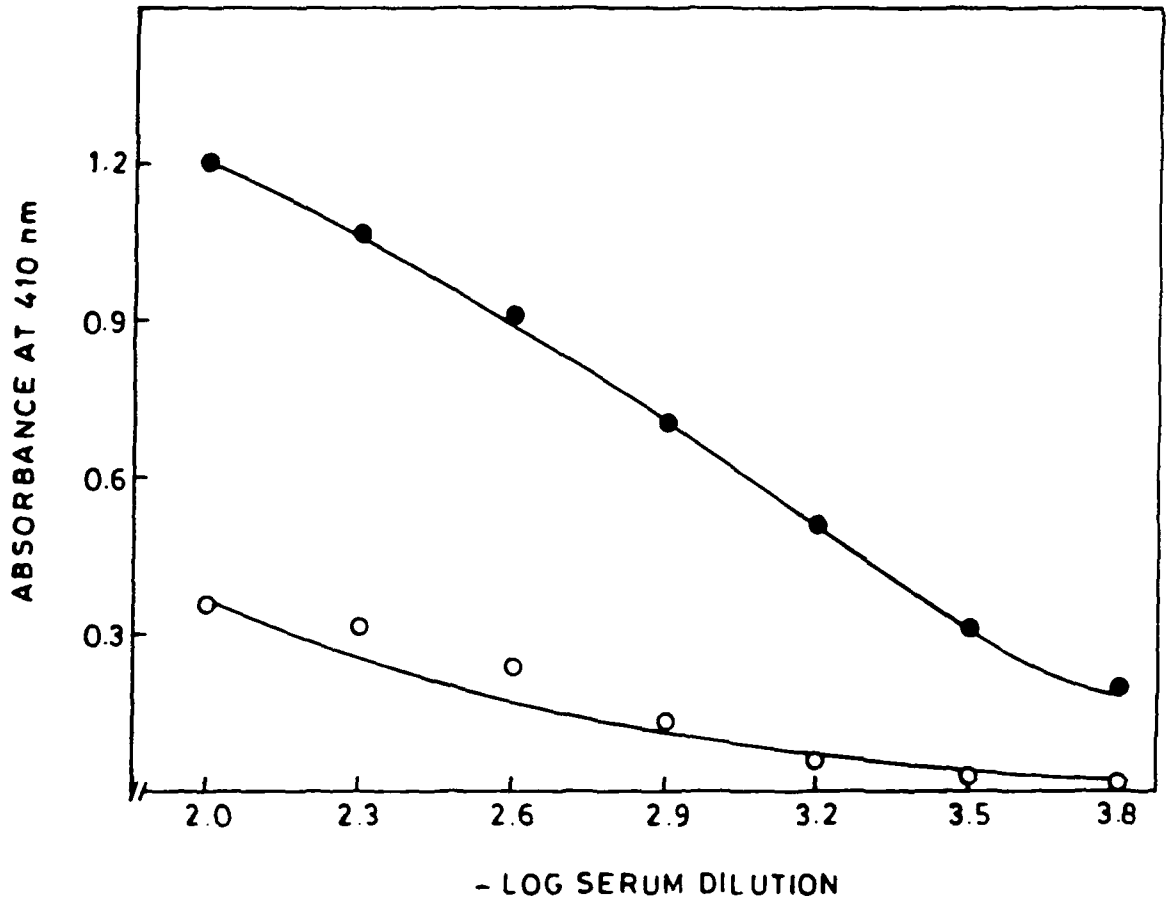


Fig.28. Direct binding ELISA of anti-DNA-BSA antibodies. The wells were coated with DNA-BSA. Immunized (—●—) and preimmune (—○—) serum.

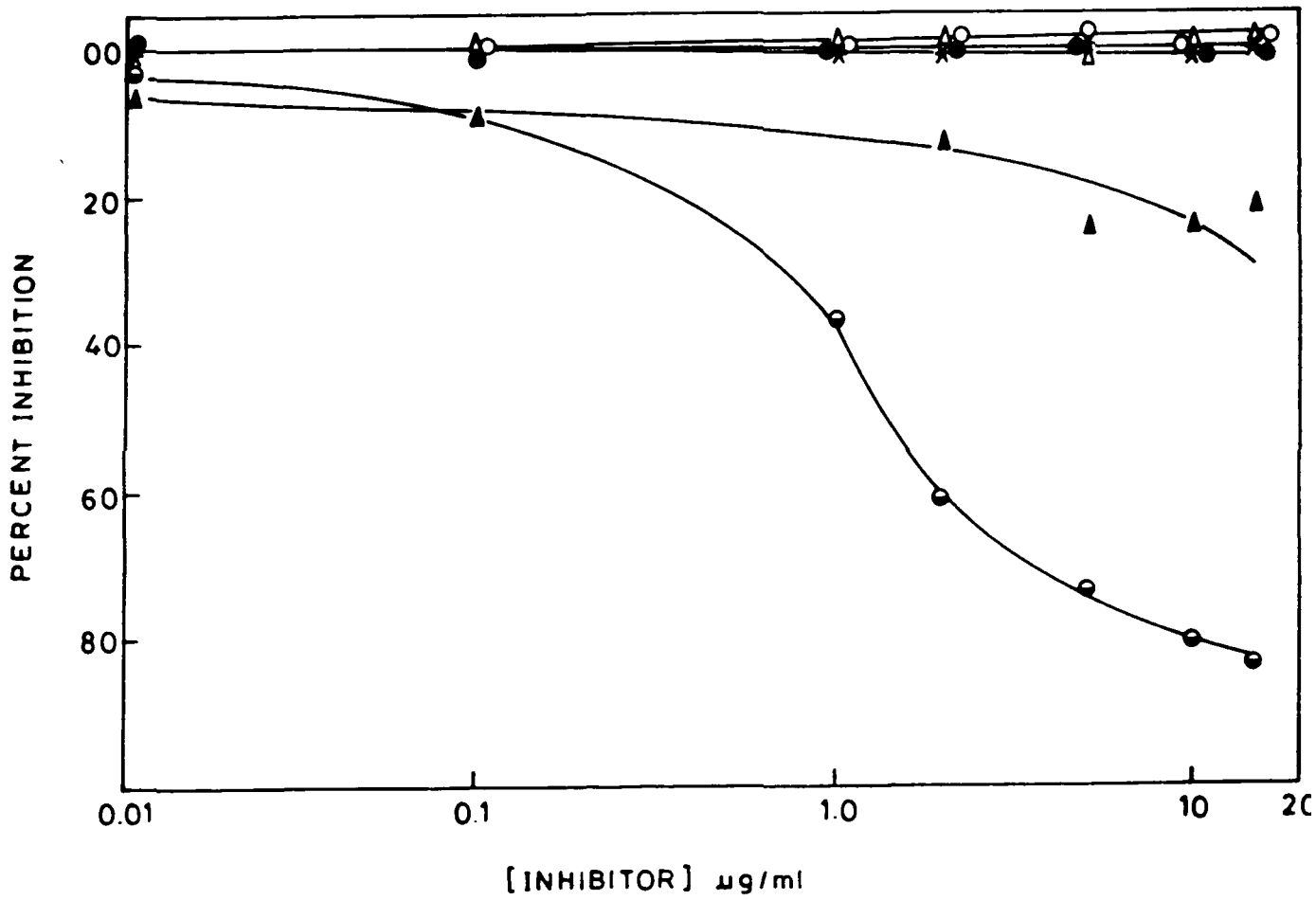


Fig.29. Inhibition of anti-DNA-BSA antibody activity by poly(rG).poly(dC) (—●—), DNA-BSA(—◒—), native DNA(—○—), ssDNA(—△—), RNA(— * —) and histone [H_2A] (—▲—).

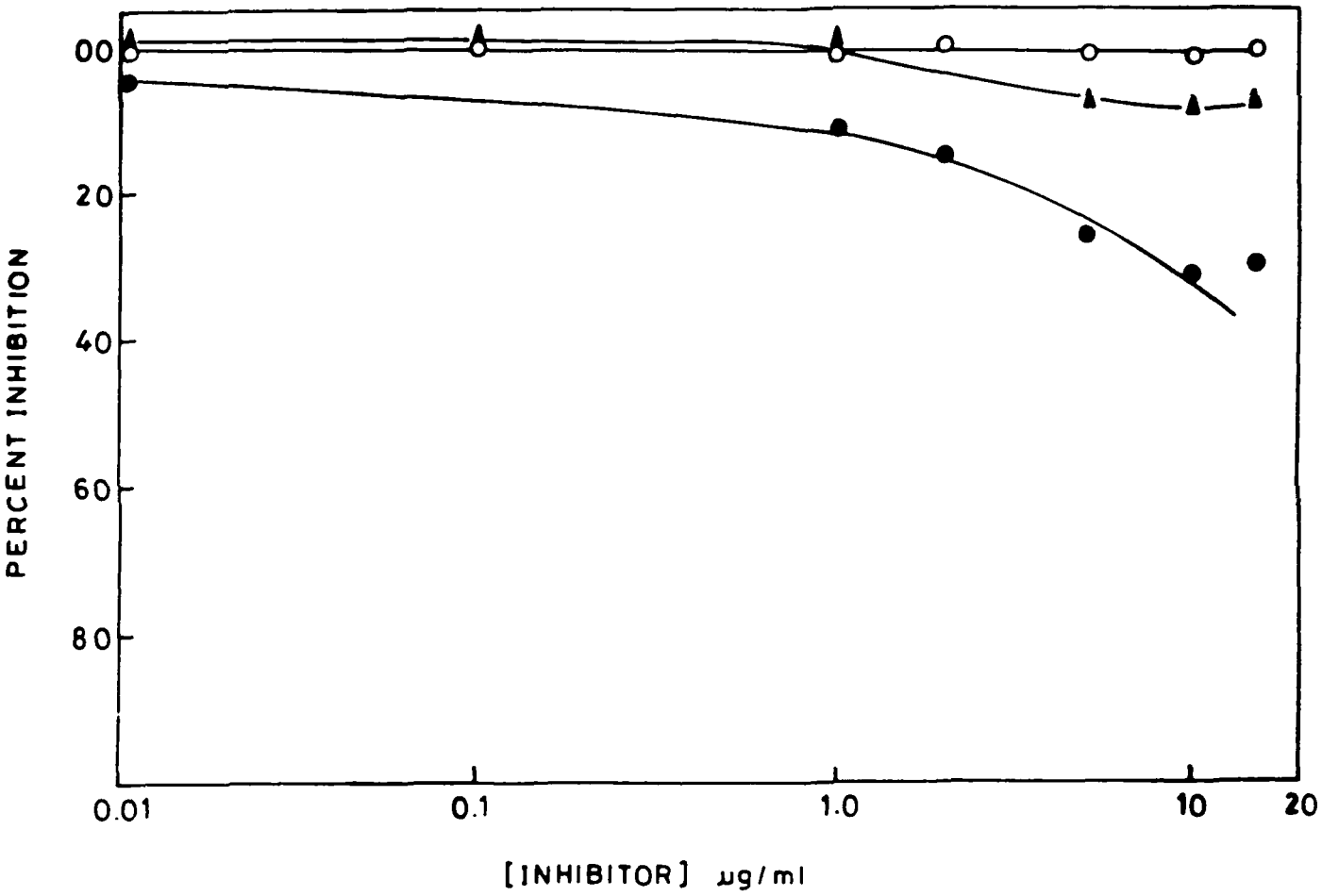


Fig.30. Inhibition of anti-DNA-BSA antibodies. The competitors were E_2-BSA-DNA(—○—), E_2-BSA (—○—), BSA(—●—) and Br-DNA(—▲—).

show almost similar binding of anti-DNA antibodies to native and Z-form of calf thymus DNA (Figs. 31 & 32). In competition-inhibition experiments, when compared with native DNA, the Br-DNA appeared equally effective inhibitor of anti-DNA antibody binding to native DNA (Figs. 33 & 34).

SLE Autoantibody Binding to Spermine-DNA Complex

Of all the polyamines, spermine causes maximum perturbation in nucleic acid conformation. Though the B→Z transition of synthetic polynucleotides in the presence of spermine has been documented (Thomas and Messner, 1988; Thomas et al., 1990), no substantial effort has been made to probe the B→Z transition of calf thymus DNA. The polyamine induced Z-form is also significant because it is achieved in physiological conditions of saline. The spermine induced Z-DNA also showed very high degree of recognition for SLE autoantibodies both in direct binding and competition ELISA (Figs. 35 & 36).

Recognition of Estradiol-BSA-DNA Conjugate by SLE Autoantibodies

Three SLE sera having high titer anti-DNA antibodies (>1:6400) with preference for native DNA were obtained from patients falling under the criteria laid down by American Rheumatism Association for this disease. Estradiol-albumin-DNA conjugate, DNA-BSA conjugate and native DNA were

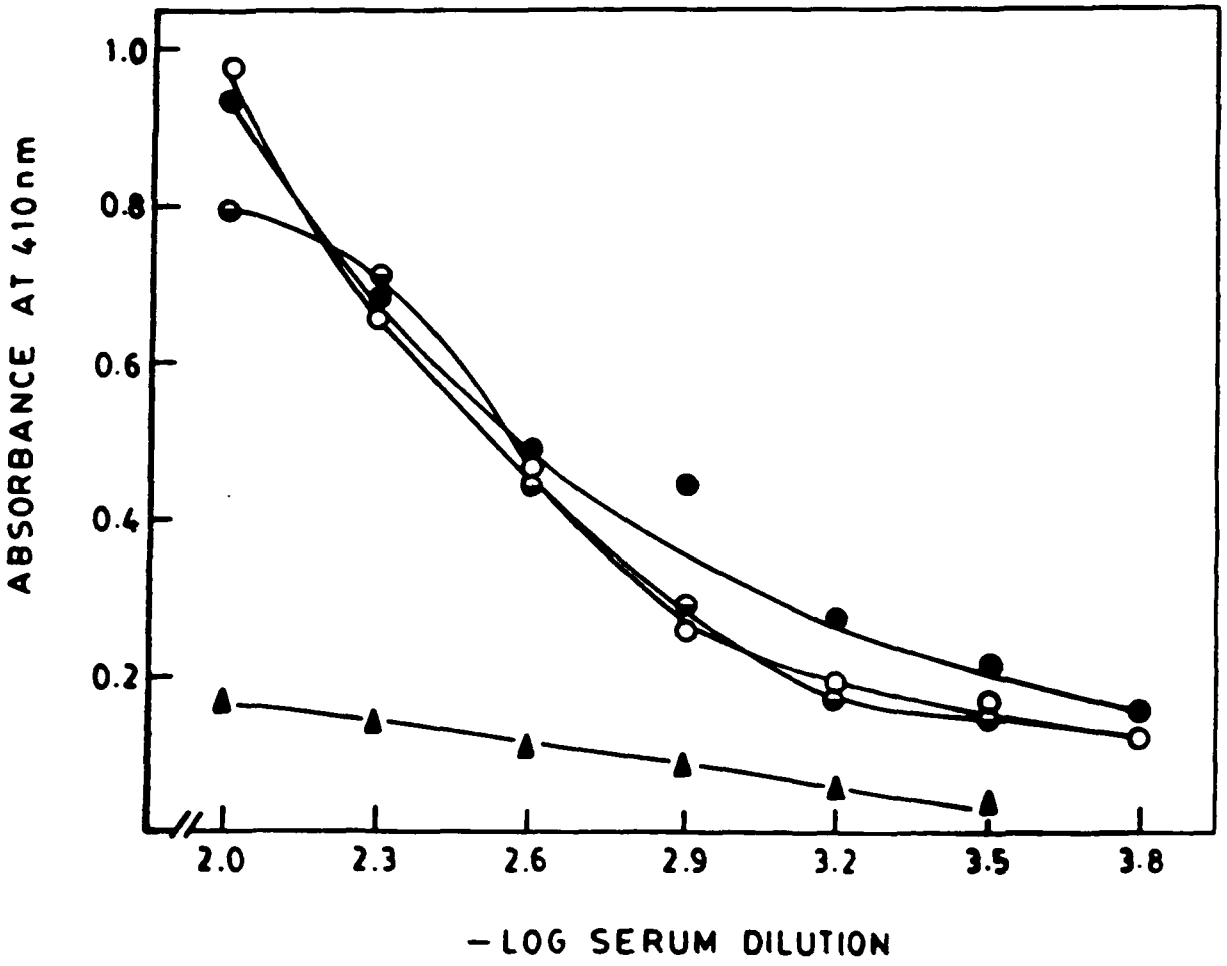


Fig.31. Direct binding assay of anti-DNA autoantibodies. The plate was coated with native DNA. Normal human serum (—▲—), SLE sera (—●—;—◐—;—○—).

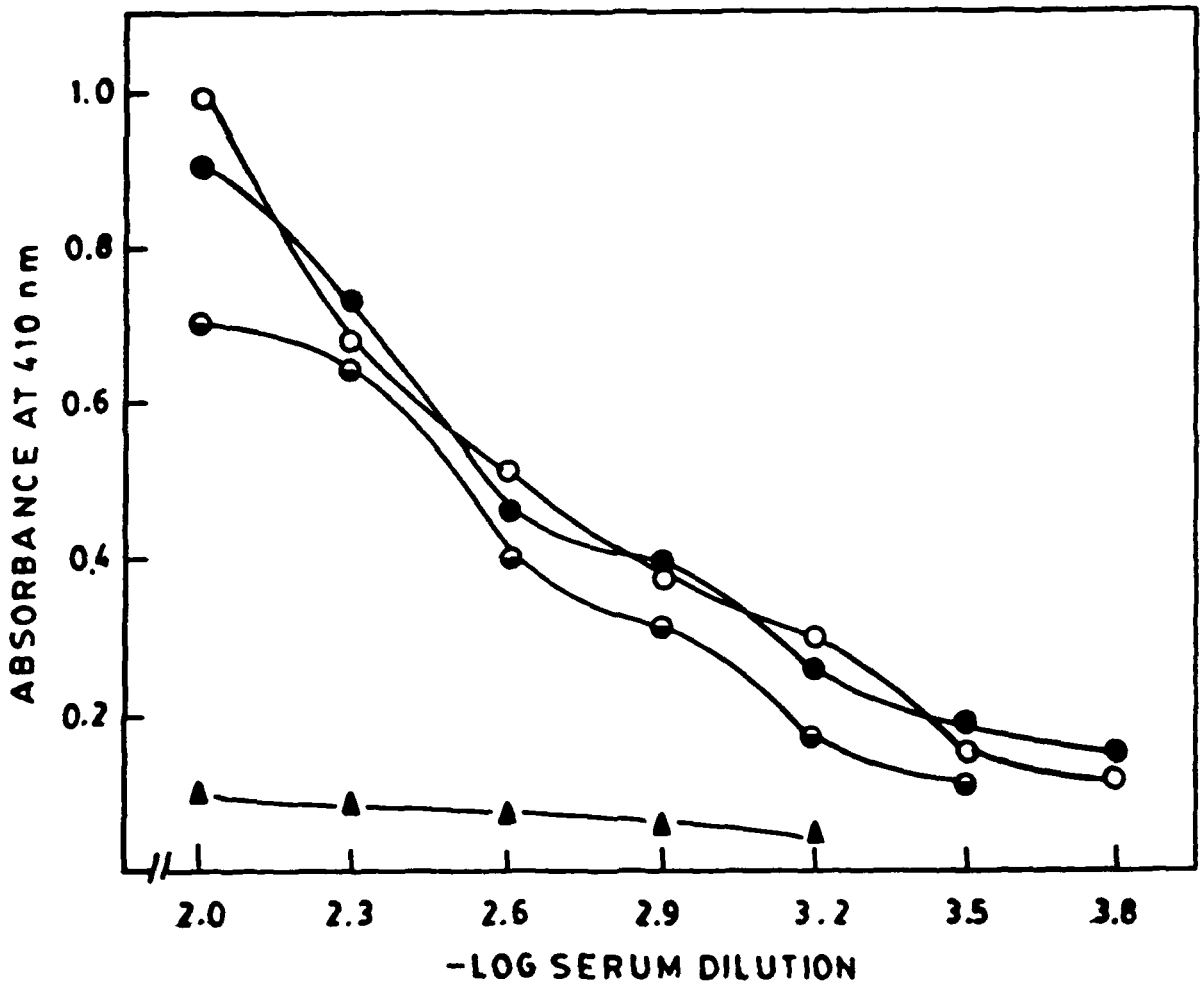


Fig.32. Direct binding assay of anti-DNA autoantibodies on the plate coated with Br-DNA. Normal human serum (—▲—), SLE sera (—●—;—◐—;—○—).

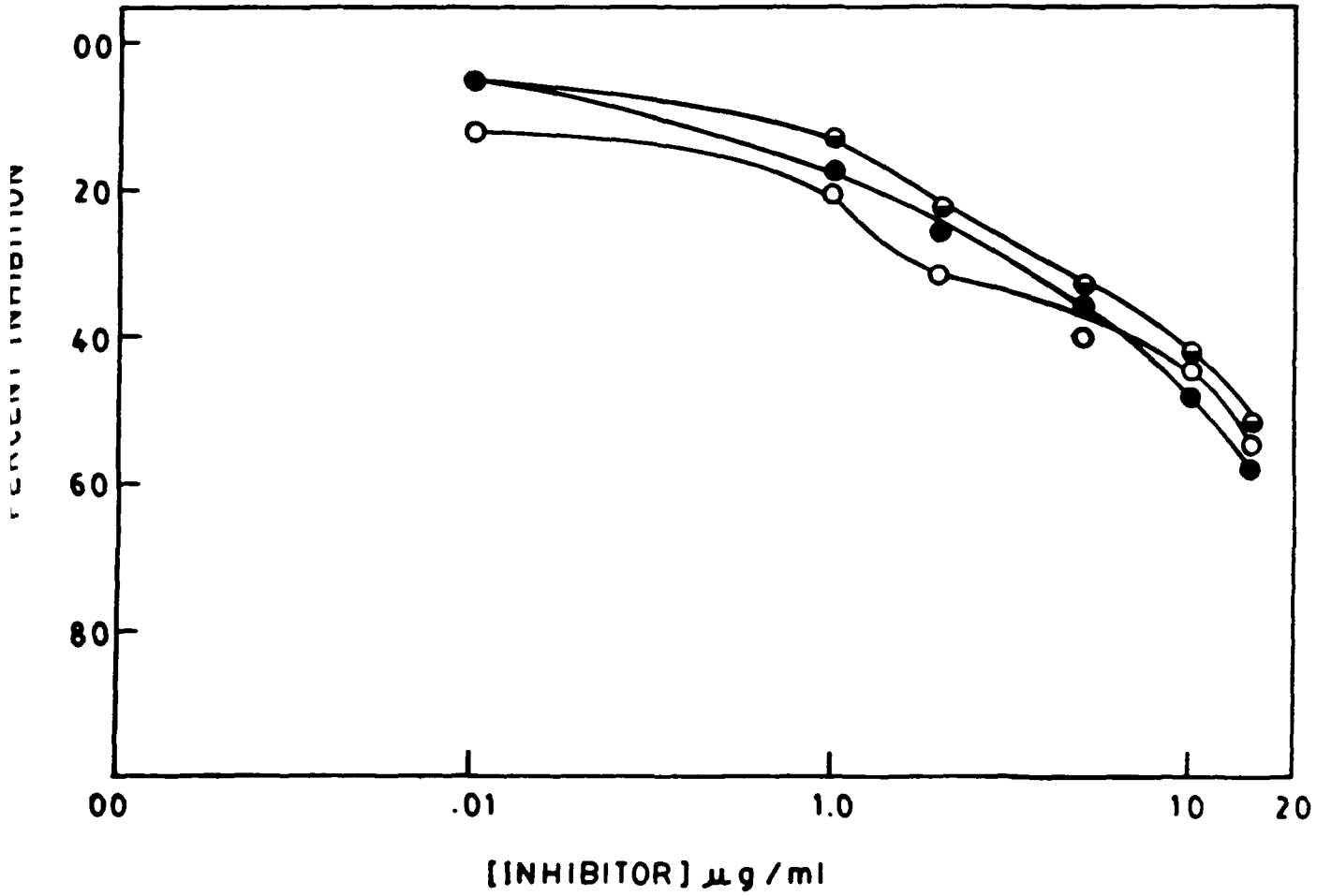


Fig.33. Inhibition of SLE anti-DNA antibodies by native DNA. The plate was coated with native DNA.

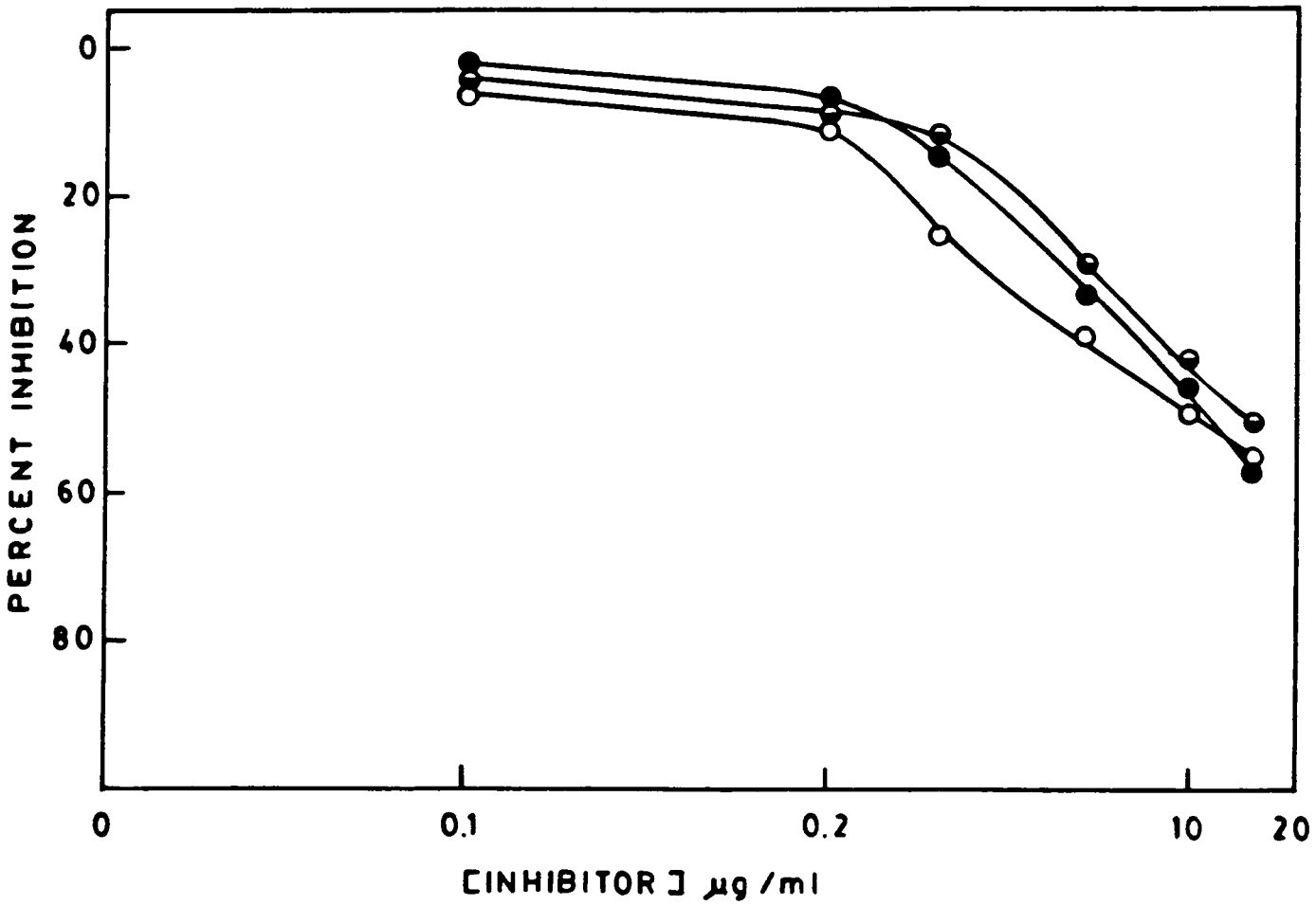


Fig.34. Inhibition ELISA of SLE anti-DNA antibodies. The competitor was brominated DNA and the coating antigen was native DNA.

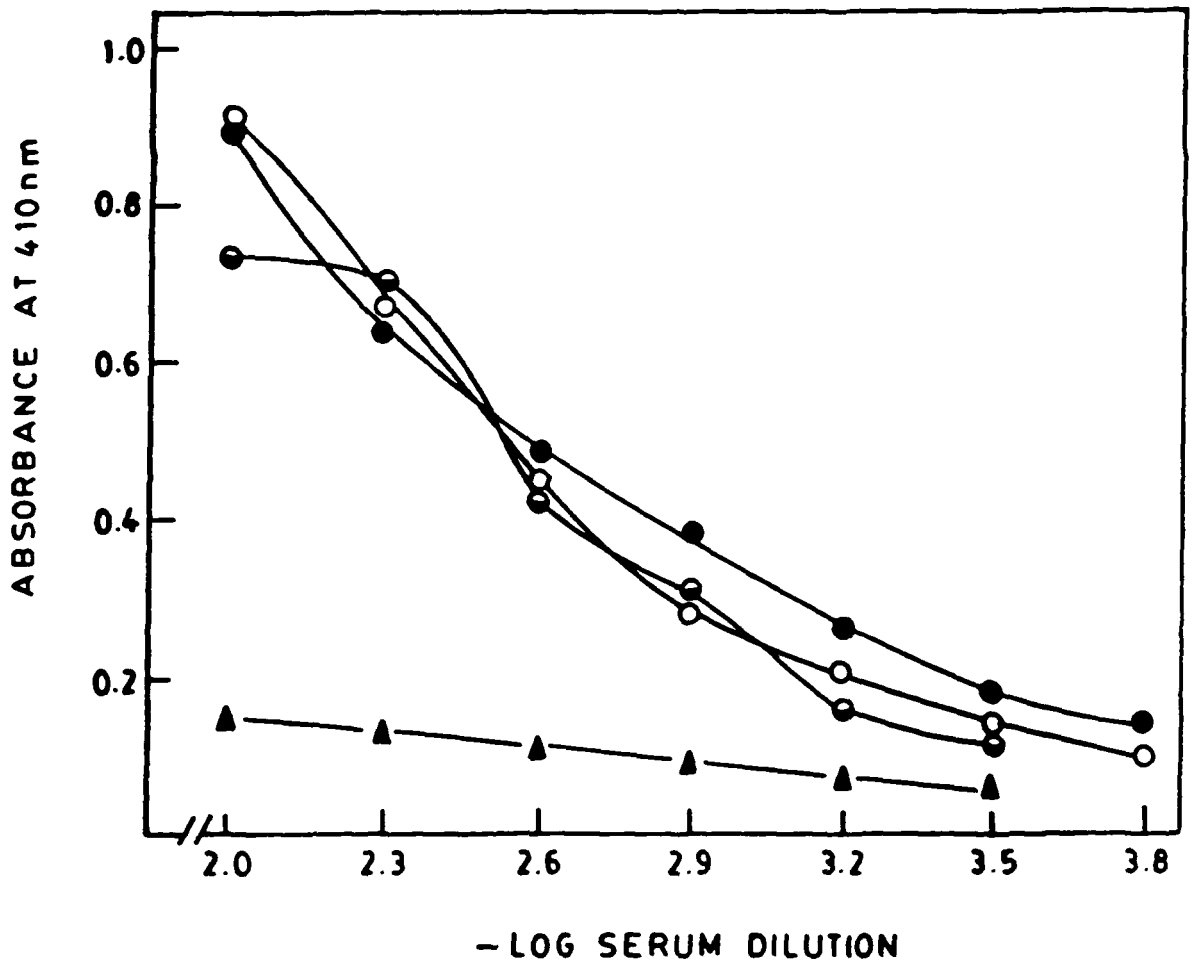


Fig.35. Direct binding assay of anti-DNA antibodies on the plate coated with DNA-spermine complex. Normal human serum (—▲—), SLE sera (—●—;—◐—;—○—).

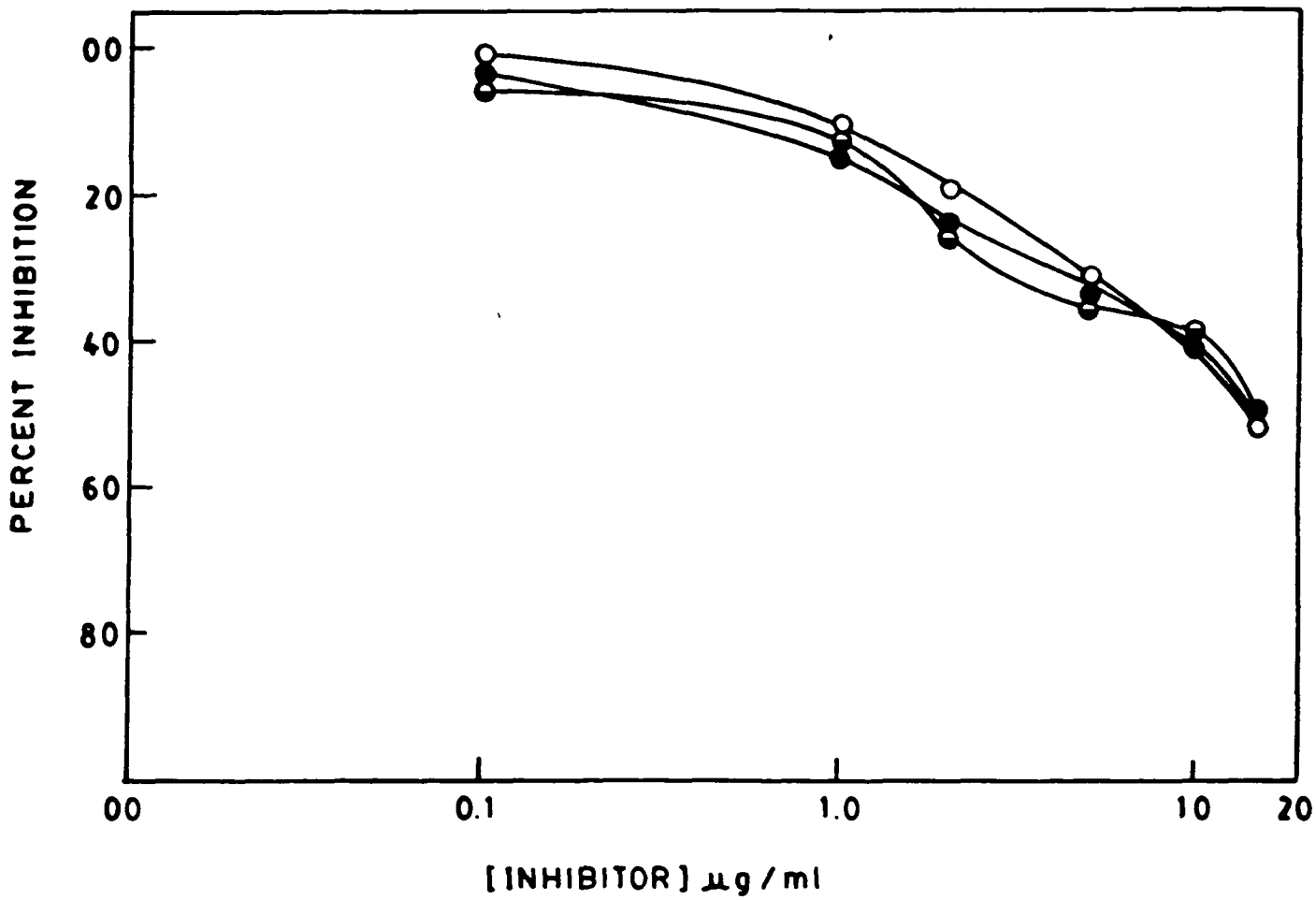


Fig.36. Inhibition of SLE anti-DNA autoantibodies by DNA-spermine complex. The plate was coated with native DNA.

employed as competitors of antibody binding to the native DNA coated on polystyrene plate. A high degree of inhibition of SLE anti-DNA antibodies was observed with E_2-BSA-DNA conjugate (Fig. 37) at an inhibitor concentration of 15 µg ml^{-1}. This signifies the recognition of SLE anti-DNA auto-antibodies by E_2-BSA-DNA conjugate. The binding of SLE autoantibodies to native DNA and E_2-BSA-DNA was also assessed by gel retardation assay. When mixtures of SLE anti-DNA antibodies (IgG) and native DNA were subjected to agarose gel electrophoresis the complexes showed different degree of retarded mobility (Fig. 38a). With increasing IgG concentration the amount of immune complexes formed also increased as revealed by fluorescence intensity, whereas the amount of unbound DNA showed a proportional decrease. When complexes of SLE IgG and E_2-BSA-DNA were electrophoresed on agarose gel under identical conditions, the retarded mobility as a result of immune complex formation was again observed (Fig. 38b). This conclusively shows the binding of SLE anti-DNA antibodies to E_2-BSA-DNA.

Human Autoantibody Binding to Estradiol

Naturally occurring SLE autoantibodies with specificity for native DNA were checked for their binding with ß-estradiol using colorigenic and fluorogenic substrates. An SLE serum having high titer anti-DNA antibody activity

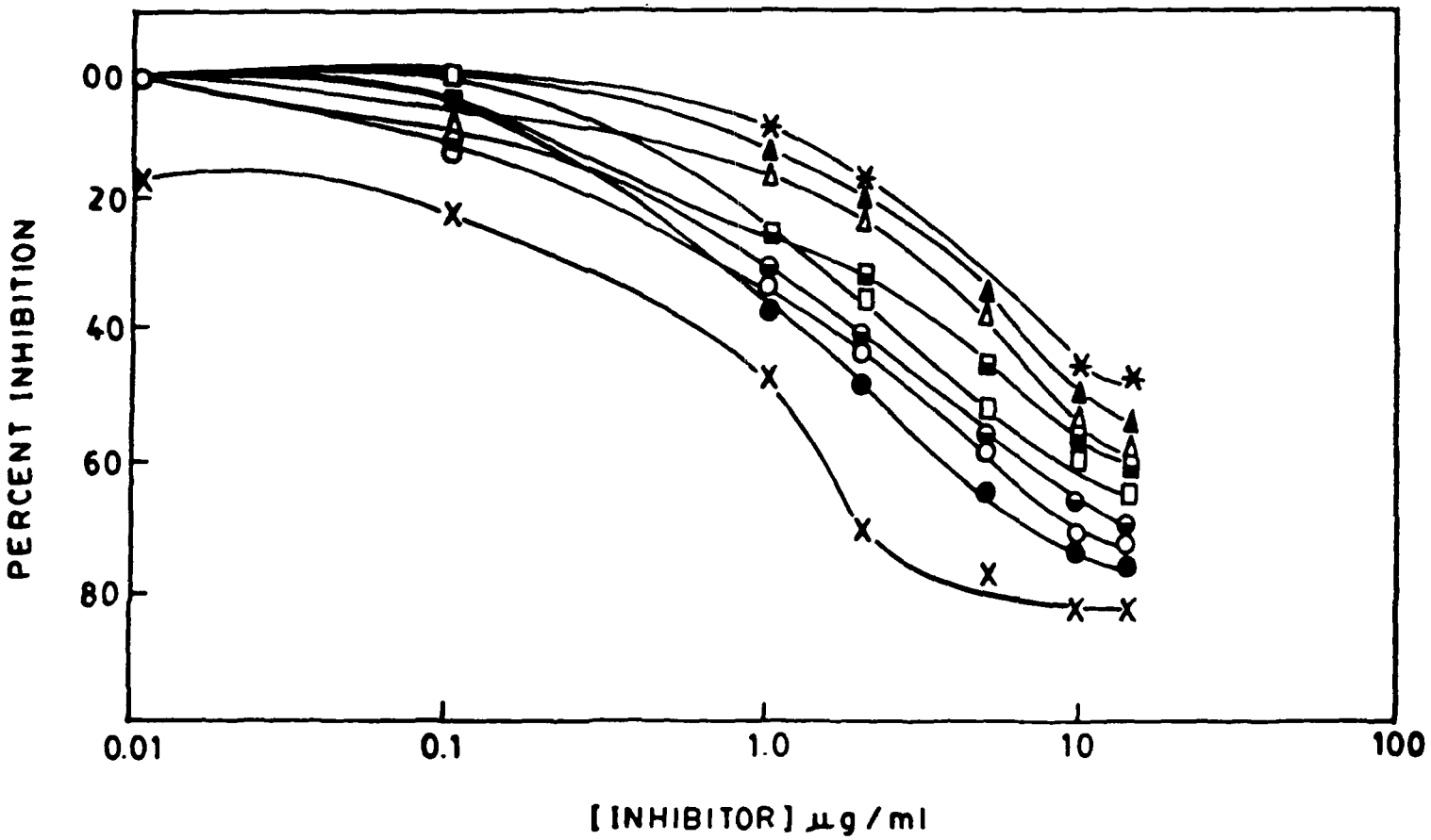
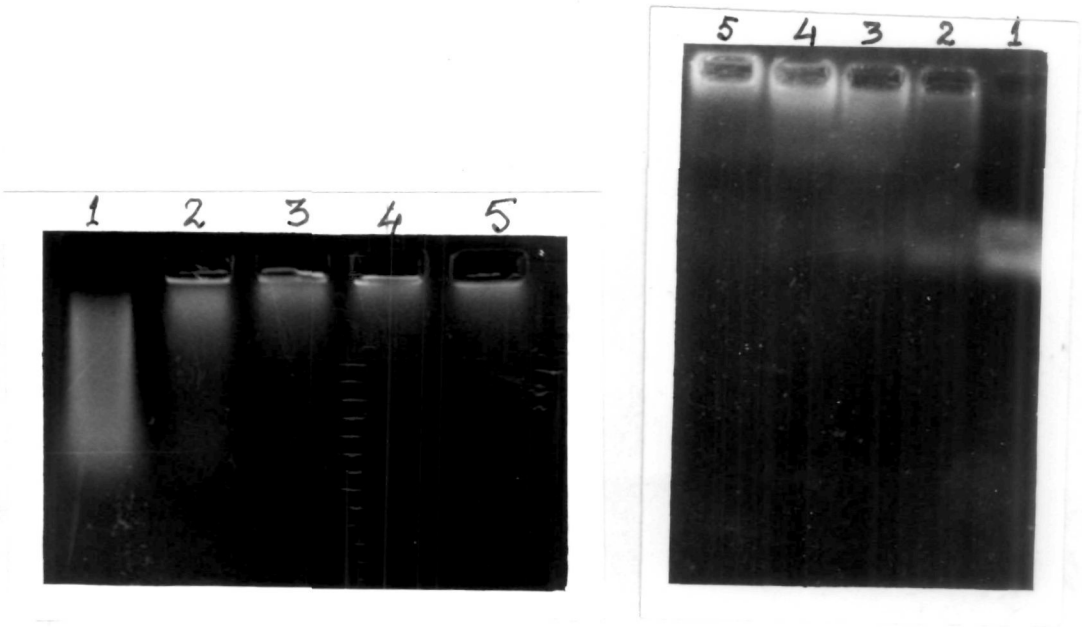


Fig.37. Inhibition of SLE anti-DNA antibody binding by native DNA (-X-;-○-;-△-), DNA-BSA (-□-;-■-;-✱-) and E_2-BSA-DNA (-●-;-◒-;-▲-). The polystyrene plate was coated with native DNA.



(a) (b)

Fig.38. **(a)** Binding of SLE IgG to native DNA as analyzed by gel retardation assay. Native calf thymus DNA (0.2 µg) was incubated with buffer (lane 1), and with varying concentrations of SLE IgG (5µg, lane 2; 10µg, lane3; 15µg, lane4; 20µg, lane5). The complex was electrophoresed for 2 hr on 1% agarose.
(b) Binding of SLE IgG to E_2-BSA-DNA. The conjugate (0.2 µg) was incubated with buffer (lane 1) and with various concentrations of SLE IgG (5µg, lane2; 10µg, lane3; 15µg, lane4; 20µg, lane5). The complex was electrophoresed for 2 hr on 1% agarose.

exhibited enhanced recognition of ß-estradiol as compared to native DNA in ELISA experiments (Fig. 39). Six more SLE sera were checked for their binding with native DNA and ß-estradiol using colorigenic and fluorogenic substrates. It has been reported that the fluorogenic substrate increases the sensitivity of ELISA (Ali and Ali, 1983). Figures 40 and 41 show the enhanced binding of SLE anti-DNA autoantibodies with ß-estradiol as compared to native DNA at 1:100 serum dilution in direct binding ELISA performed using PNP-P and 4MU-P as the respective enzyme substrates.

To further probe the binding of SLE anti-DNA antibodies with ß-estradiol, SLE autoantibodies were affinity purified on DNA-Sepharose column. SLE sera were diluted 1:10 in PBS and passed through the column. After application of linear sodium chloride gradient the bound material eluted in two peaks (Fig. 42). Colorimetric estimations and UV measurements revealed protein in peak 1 and DNA in peak 2. The absorbance ratio (A_{278}/A_{251}) of peak 1 fractions was found to be 2.47, typical of mammalian IgG. The affinity purified SLE IgG also showed enhanced binding with ß-estradiol as compared to native DNA using PNP-P as enzyme substrate (Fig. 43).

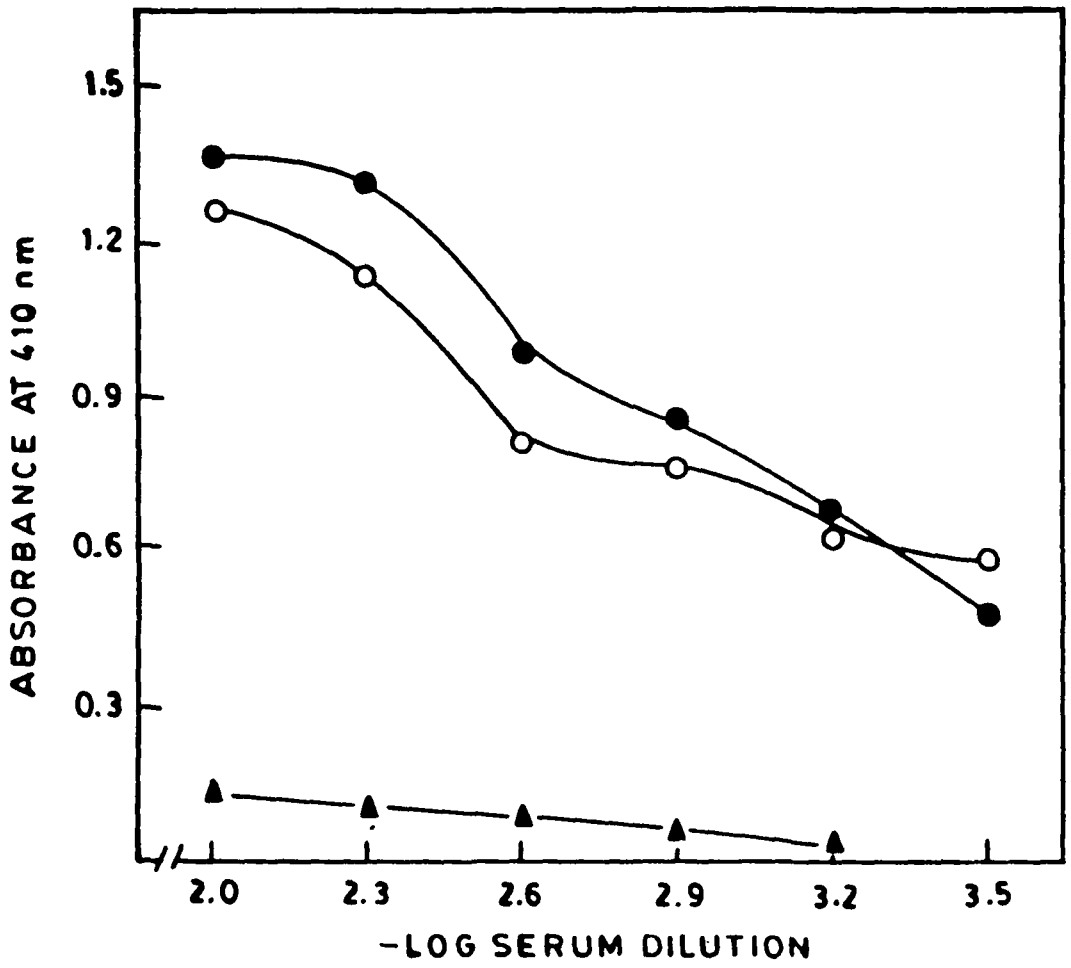


Fig.39. Direct binding assay of SLE autoantibodies. The plate was coated with native DNA (–○–) and ß-estradiol (–●–). Binding with normal human serum (–▲–).

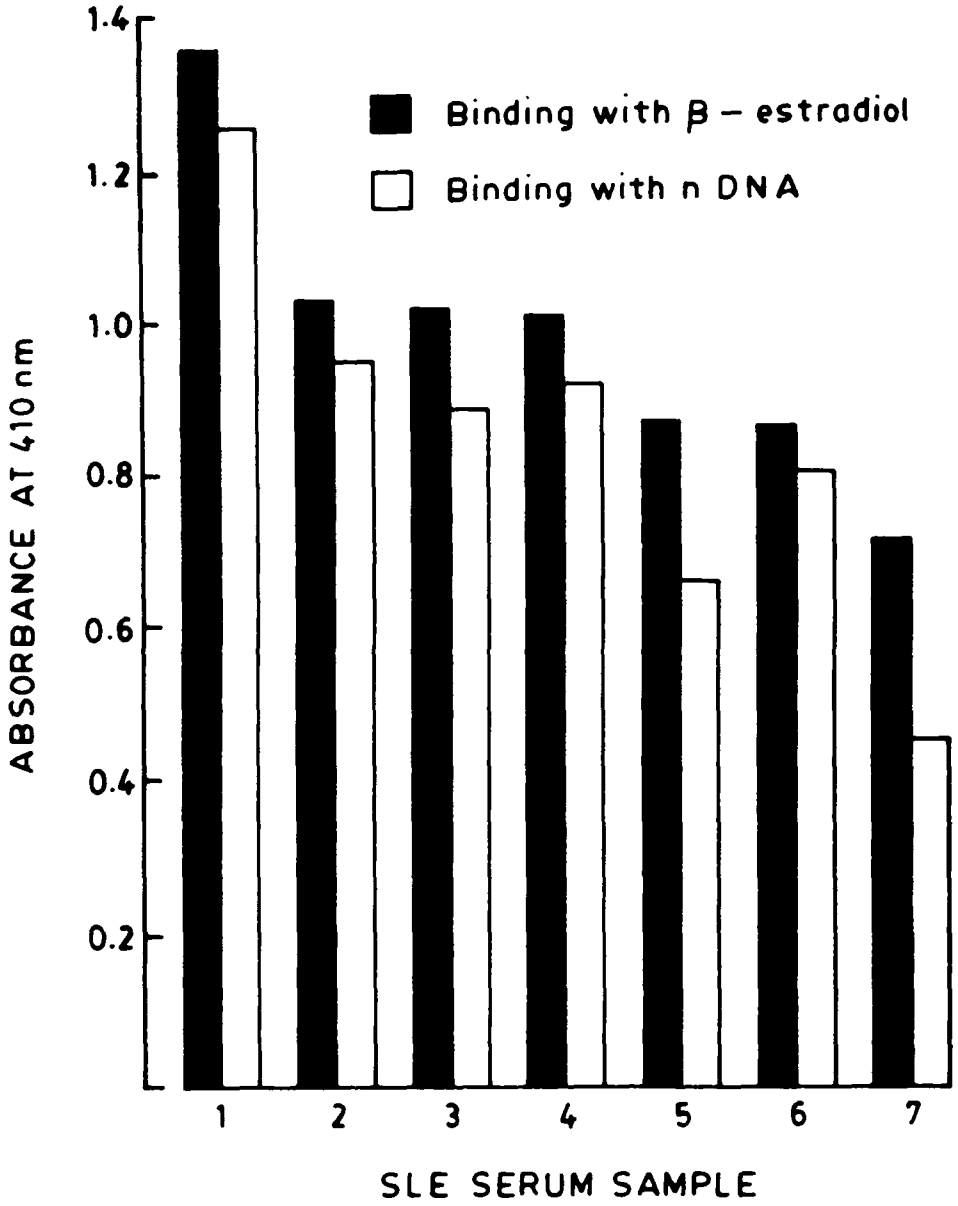


Fig.40. Binding of human lupus antibodies to native DNA (—□—) and ß-estradiol (—■—). Para-nitrophenyl-phosphate was used as enzyme substrate.

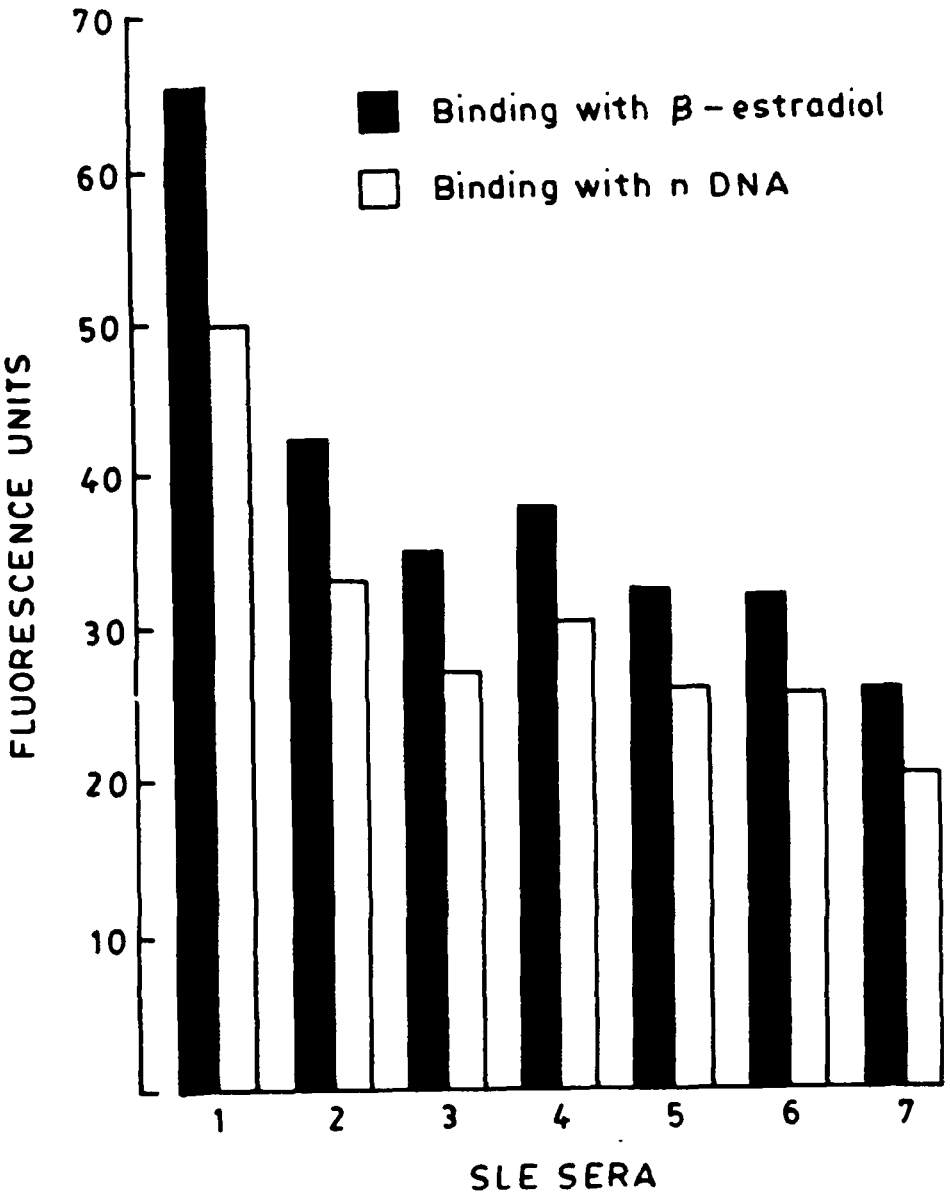


Fig.41. Binding of human SLE anti-DNA antibodies to native DNA (-□-) and ß-estradiol (-■-). The ELISA was performed using 4-methyl umbelliferyl phosphate as enzyme substrate.

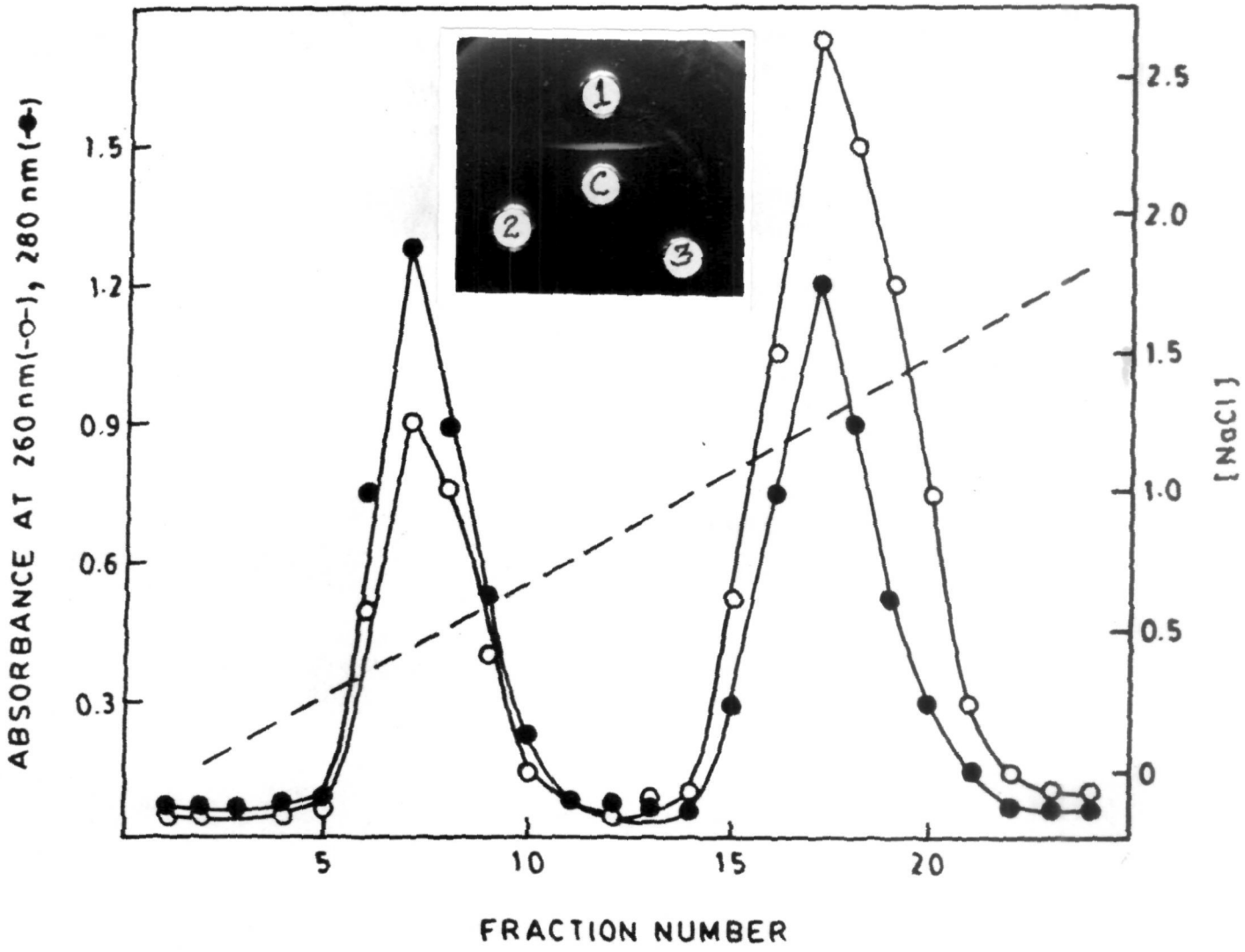


Fig.42. *Immunoaffinity purification of anti-native DNA antibodies on DNA-[polylysyl-Sepharose 4B] column.* Anti-DNA positive SLE serum was passed through the column and bound material was eluted with linear gradient (-----) of sodium chloride (0.15 to 3M) in 0.01 M Na-Pi buffer pH 7.4. **Inset** shows the results of Ouchterlony immunodiffusion. The central well (c) contained anti-human IgG. whereas peripheral wells 1, 2 and 3 contained peak 1, peak 2, and serum albumin.

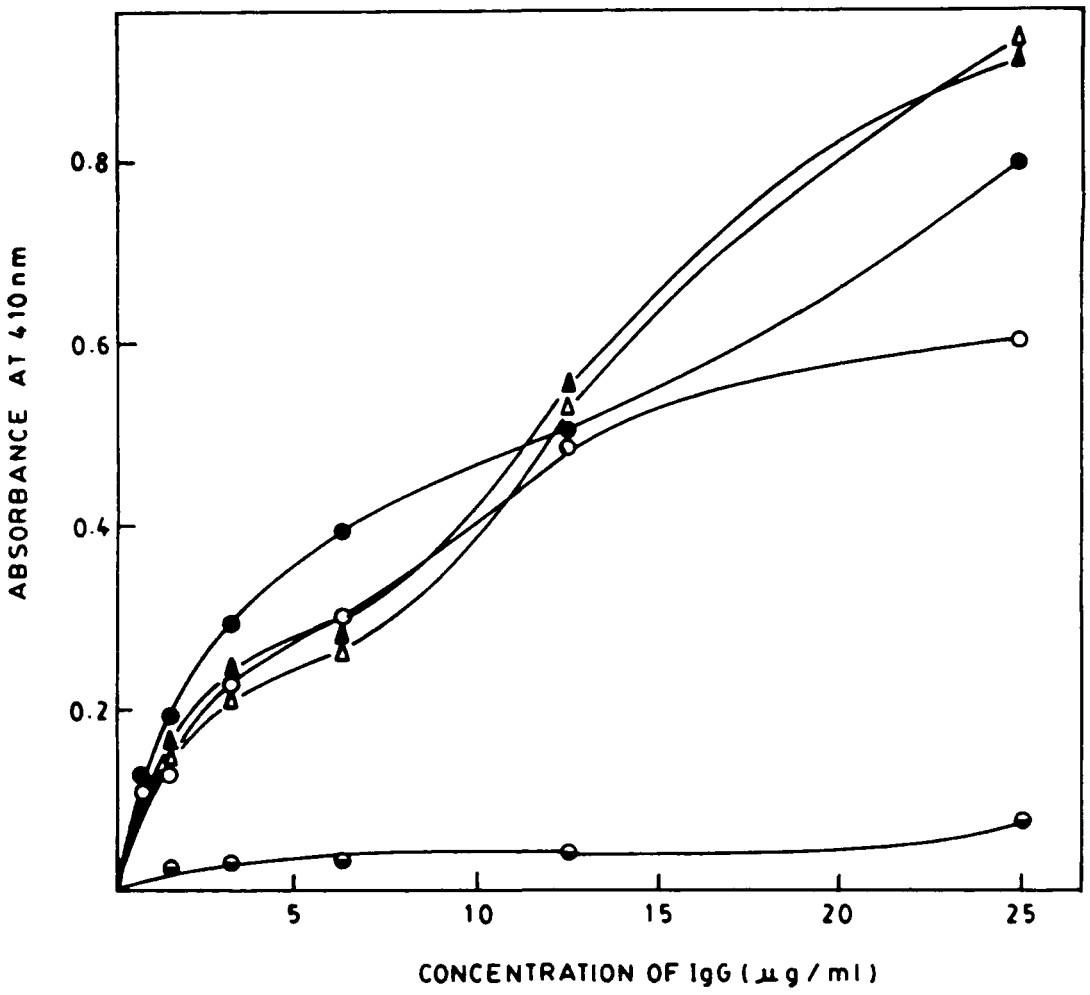


Fig.43. Binding of affinity purified SLE IgG with native DNA (—○—;—△—) and ß-estradiol (—●—;—▲—).

Discussion

Autoimmune diseases have been associated with a myriad of immunological abnormalities ranging from increased proinflammatory cytokines, decreased anti-inflammatory cytokines, increased expression of major histocompatibility complex molecules, skewing of T-cell receptor repertoire, increased numbers of B cells, and autoantibody production. It has been widely suspected that underlying all these immunological changes, there is a defect in the ability to eliminate self reactive T cells or B cells. Although much knowledge has accrued regarding various mechanisms that affect autoimmune disease, the exact causes and mechanisms of malfunction and tissue damage remain unclear. This failure is probably due to complex processes involved in immune recognition and regulation. Though a host of components that could lead to autoantibody production have been discovered, it appears that it will be the unraveling of the exact way in which these species interact that may actually lead to a complete understanding of autoimmune processes. The identification of the inciting antigen has been masked by three areas of ambiguity. First, it is uncertain whether identified autoantibodies and corresponding antigens have anything to do with the primary cause or pathogenesis of a given disease. Second, the immune diversity of autoantibodies observed in some diseases causes considerable difficulty in the identification of the

inciting antigen. Third, there is uncertainty as to whether the initial trigger has any relation to the antigen recognized by the autoantibodies (Plotz, 1992; Mongey and Hess, 1993; Levinson, 1994; Mountz et al., 1994; Radic and Weigert, 1994 and Theofilopoulos, 1995).

The presence of antinuclear antibodies in the sera of patients suffering from various autoimmune disorders is of considerable importance. These naturally occurring antibodies not only provide diagnostic/prognostic parameters to clinicians but give molecular biologists valuable tools for the better understanding of cellular processes (Woodruff et al., 1986; Pisetsky et al., 1990). Amongst these antibodies those specific for DNA (anti-DNA antibodies) have received much attention as they are highly characteristic for SLE. Efforts to induce anti-DNA antibodies by immunization with native DNA have been unsuccessful indicating thereby that native DNA *per se* is not the immunogen for these antibodies.

Despite considerable work that has been done to understand the pathogenesis of SLE, it is still unknown whether a single factor or more than one factor participate to initiate SLE (Frank et al., 1990). The polyspecific binding of SLE autoantibodies to a whole gamut of modified nucleic acid conformers (Ali et al., 1991; Alam and Ali, 1992; Alam et al., 1992., 1993; Arjumand and Ali, 1994;

Klinman et al., 1994, and Moinuddin and Ali, 1994) has frustrated the efforts of lupus researchers to understand the origin and consequences of anti-ds DNA antibodies. It has long been recognised that more active immune responses and a higher incidence of autoimmune diseases occur in females (Lahita, 1985). Sex appears to be a potent risk factor as suggested by strong preponderance of SLE in females as compared to males, thus drawing immediate attention to the possible involvement of female sex hormones (Talal and Ahmad, 1987; Folomeev et al., 1990, 1992; Lahita, 1992a, 1992b). Estrogen has also been reported to be a potent disease accelarator in SLE prone MRL lpr/lpr mice (Carlsten et al., 1990). Furthermore, elevated binding of estradiol to serum globulins was found in SLE patients and women who had taken oral contraceptives as compared to normal men and women (Counihan et al., 1991).

In this study, two aspects were probed. One, the induction of Z-conformation in native calf thymus DNA as a result of bromination in high salt and by treatment with polyamines and second, the covalent modification of native DNA (average size 200 bp) by linking it to BSA and estradiol-albumin (E_2-BSA)respectively. The possible role of these modified conformers in the pathogenesis of SLE has been investigated.

The B→Z transition of synthetic polynucleotides as a

result of bromination in high salt has been well documented (Thomas et al., 1988). Bromination of double stranded DNA altered its UV absorption characteristics. The changes were more pronounced in the case of native DNA brominated in high salt compared to the polymer brominated in low salt. A large decrease in absorbance at 260 nm and increased absorbance at around 295 nm was observed in DNA brominated in high salt, while the polymer brominated under physiological conditions of saline did not show such marked changes in the UV spectrum. Moreover, the absorbance at around 300 nm in the difference spectrum was quite enhanced for native DNA brominated in high salt compared to the low salt sample. The spectra obtained in the case of DNA brominated in 4M NaCl are quite similar to the pattern exhibited by poly(dG-dC).poly(dG-dC) when brominated in high salt, indicating thereby that native calf thymus DNA has attained Z or Z-like conformation. The absorbance ratio (A_{295}/A_{260}) of native DNA brominated in high salt was 0.35 while for the low salt brominated form, it was 0.188. This is also a parameter to judge the formation of Z-conformation because A_{295}/A_{260} value for prototype Z-DNA is 0.3 and this value has been accepted as a characteristic of Z-conformation (Thomas and Strobel, 1988). The results were also evaluated by circular dichroism studies wherein the inversion of spectrum in the case of DNA brominated in high salt provides additional

evidence for the attainment of Z-conformation.

The barrier of interconversion of double stranded DNA between right handed and left handed helical forms was also challanged by polyamines, which are known to induce Z-conformation in synthetic polynucleotides (Thomas et al., 1990; Takeuchi et al., 1991). The importance of spermidine and spermine lies in their requirement for normal cell growth and differentiation. Their interaction with nucleic acids may be responsible, in part, for the biological function of polyamines. (Pegg, 1988). The transition of native B-form of DNA to Z-conformation in the presence of polyamines was checked by absorbance ratio and interaction with anti-Z-DNA antibodies. Taking into consideration the absorbance ratio (average 0.315) observed in case of poly(dG-dC).poly(dG-dC) and concentration of spermine required to achieve this conversion (40 µg/ml), native calf thymus DNA indicated around 15% isomerization into Z-conformation (average absorbance ratio was 0.125 at 100 µg/ml of spermine). The specific binding of anti-Z-DNA antibody to polyamine-DNA complex is another evidence of conformational isomerization of native B-epitopes into Z-conformation in the presence of increasing concentrations of spermine, spermidine and putrescine. Poly(dG-dC).poly(dG-dC) taken as a reference polymer showed similar epitope polymerization. Anti-Z-DNA antibody had no binding with

poly(dG-dC).poly(dG-dC) and native DNA when the polynucleotides were coated on the microtitre plate in the absence of polyamines. Monoclonal and polyclonal anti-Z-DNA antibodies have been used to detect the presence of left handed segments in natural DNAs (Arndt-Jovin et al., 1983; Lee et al., 1984; Nordheim et al., 1986). The solid phase enzyme immunoassay is a very sensitive technique to study the B->Z conformational transition of polynucleotides (Thomas et al., 1988).

Native calf thymus DNA (average size 200 bp), obtained as a result of controlled digestion with micrococcal nuclease was covalently modified by linking with E_2-BSA in order to see whether the female sex hormone, when conjugated to DNA, could form a possible antigenic trigger for SLE as the disease is more prevalent in women. The linked and unlinked species were separated by exclusion chromatography on Sephadex G-200 column. Two distinct peaks were obtained. However, colorimetric estimations could detect DNA only in the first peak while protein was present in both the peaks, implying that peak 1 contained E_2-BSA-DNA conjugate. The altered spectral properties of DNA signify modifications in the double helix as a result of conjugate formation.

The change in the absorbance ratio (A_{260}/A_{280}) from 2.2 in case of DNA to 1.6 for DNA-BSA conjugate and 1.45 for E_2-

BSA-DNA conjugate also suggests DNA modification as a result of conjugation.

The modified nucleic acid polymers, with the exception of brominated DNA, because in this case the helix did not melt even at 100°C, were also characterised by thermal denaturation studies. While for native DNA the observed Tm was 87°C, it was 90°C in the case of DNA-spermine complex. Moreover the DNA-spermine helix disruption started quite late (87.5°C) compared to native DNA. This means that the helix was resisting heat induced strand separation to a greater extent in case of DNA-spermine, meaning thereby that complexation of spermine has imparted stability to the DNA molecule.

The E_2-BSA-DNA and DNA-BSA conjugates were also characterised by thermal denaturation studies. The increased Tm value and decreased percent denaturation at 80°C in case of E_2-BSA-DNA as compared to native DNA fragment indicates that E_2-BSA-DNA is thermodynamically more stable than the corresponding native DNA fragments. On the contrary, the DNA-BSA conjugate was found to be less stable as judged by the decreased melting temperature and greatly increased percent denaturation at 80°C. The only difference between DNA-BSA and E_2-BSA-DNA conjugate is the presence of estradiol molecule linked to BSA in the later species. Thus inference can be drawn that estradiol is somehow imparting

stability to the helix in E_2-BSA-DNA conjugate.

Additional evidence for the structural perturbations in native DNA and 200 bp DNA fragments as a consequence of interactions with protein, polyamine and hormone was gathered by computation of thermodynamic parameters. Native DNA displayed a large negative value for Gibb's free energy of denaturation (ΔG_D) till 85°C. The results depicted in Table 7 speculate the tremendous stability exhibited by double helical native DNA. Shift in ΔG_D value from negative to positive above 85°C suggest the disruption of Watson-Crick base pairing and the transition of double helical nucleic acid from the native to denatured state.

In comparison to native DNA, the DNA-spermine complex displayed negative ΔG_D values beyond 85°C suggesting that DNA-spermine complex is thermodynamically more stable than native DNA. This could be attributed to the fact that polyamine (spermine) having multiple positively charged amino groups interacts electrostatically or ionically with the negatively charged phosphate backbone of DNA and during the course of interaction, it encompasses the DNA macromolecule thereby enhancing helix stability.

Apart from native DNA, the DNA fragments (average size 200 bp) were also employed in this study to probe their interaction with BSA and E_2-BSA respectively. In case of DNA fragments negative ΔG_D value was obtained only till 70°C

while for native DNA negative ΔG_D value persisted till 85°C implying thereby, that compared to native DNA, the 200 bp DNA fragments are thermodynamically less stable. The decreased stability of 200 bp fragments could be attributed to low G≡C contents. In case of DNA-BSA conjugate, the negative ΔG_D values were observed only till 45°C. Thus a net difference of around 25°C was observed in the persistance of negative ΔG_D values when compared with the corresponding control (200 bp DNA). The thermodynamic results speculate that a large portion in the DNA-BSA conjugate was already in the unstacked form. Clearly, the covalent conjugation of BSA with the amino groups of nucleic acid bases appears to completely obliterate the favourable A=T and G≡C pairing interactions of the double helical DNA. The instability may be attributed to the conjugation of BSA to the potential sites or substrates in DNA rendering these conjugated sites in DNA topologically less constrained than the corresponding fully paired and fully stacked Watson-Crick interactions.

However, when estradiol-BSA was covalently linked to DNA fragments (~200 bp), a totally different type of observation was recorded. In this case, surprisingly, large negative ΔG_D values were observed till 77°C of thermal supplementation. Thus, *a priori* to DNA-BSA, where destabilization was observed, entirely reversed results were

observed (tremendous stability) in the case of E_2-BSA-DNA conjugate. The thermodynamic parameters suggest that, although the glutaraldehyde aided conjugation of BSA alone resulted in helix opening, the conjugation of E_2-BSA has somehow stabilized the helix. The increased stability of this hormone-protein aducted DNA is suggestive of the formation of crosslinks in the 200 bp DNA macromolecule. The thermodynamic parameters observed here substantiate the mid-point melting (Tm) data described earlier.

The results of time dependent denaturation of native as well as modified nucleic acid were also interesting. The lag period shown by DNA-spermine complex was a bit larger as compared to native DNA indicating the induction of an additional kinetic barrier in native DNA as a consequence of ionic interaction with spermine. The increase in the $t_{1/2}$ (half life) value for the denaturation of DNA-spermine complex in comparison to native DNA further substantiates the above argument.

Native DNA fragments (average size 200 bp) exhibited a relatively shorter lag phase, thereby suggesting that the kinetic barrier for 200 bp DNA was relatively weaker than native DNA. On the contrary, DNA-BSA conjugate formed as a result of covalent conjugation by glutaraldehyde exhibited a minor transition but not the large lag period, perhaps due to the denaturation of a "segment" involving the conjugated

sites in the DNA-BSA conjugate, which consequently initiates the rapid denaturation of nucleic acid protein conjugate. The decrease in the half life ($t_{1/2}$) for melting of DNA-BSA conjugate supports the above explanations. Surprisingly, 200 bp DNA covalently linked to hormone protein (E_2-BSA) conjugate exhibited a small increment over the lag period of native DNA (~200 bp). This increase was substantial when compared to the lag period of DNA-BSA conjugate. This could be due to the introduction of a large kinetic barrier in the 200 bp DNA as a result of E_2-BSA conjugation, perhaps due to the formation of crosslinks in the helix. The increase in $t_{1/2}$ for melting of E_2-BSA-DNA conjugate further substantiates the above argument.

The E_2-BSA, DNA-BSA and E_2-BSA-DNA conjugates were found to be potent immunogens and induced high titre antibodies in rabbits. The induced antibodies were specific for their respective immunogens. The binding of anti-E_2-BSA antibodies to E_2-BSA-DNA and vice-versa clearly indicates that antibodies are recognizing common epitopes on these two antigens. The inhibition in the activity of anti-E_2-BSA and anti-E_2-BSA-DNA antibodies by estradiol is due to the presence of a small population of antibodies that is cross-reactive with estradiol. None of the three antibodies showed inhibition with either poly(rG).poly(dC) or native calf thymus DNA brominated in high salt. The data indicates that

the antibodies are not recognizing either A/A-like or Z/Z-like conformations meaning thereby that E_2-BSA-DNA and DNA-BSA conjugates have attained neither A-nor Z-conformation. The induced antibodies did not recognize native DNA. However, anti-E_2-BSA-DNA antibodies were inhibited by heat denatured DNA (ssDNA) and RNA, but at a high inhibitor concentration (75 µg/ml), pointing towards the presence of a minor population of base specific antibodies.

The recognition of various DNA conformations by SLE anti-DNA antibodies has got far reaching significance for the polyspecificity of SLE autoantibodies. The binding specificity of anti-DNA antibodies to various modified forms of DNA was analyzed by inhibition ELISA in which the ability of a competitor to block the DNA-autoantibody interaction is assessed. Human autoantibody recognition of the Z-conformation induced either as a result of bromination in high salt or by polyamines (under physiological conditions of saline) is significant because Z-DNA can arise *in vivo* as a result of various molecular events and also, the formation of Z-DNA can be facilitated by protonated amines. It is worth while to mention here that significantly elevated levels of polyamines in the case of active SLE patients (Puri et al., 1978) can, *in vivo*, lead to the attainment of Z-conformation by native DNA. Since Z-DNA is highly immunogenic, the possibility of this polymer acting as

antigen for the production of human autoantibodies cross-reactive with native DNA could be one of the factors for the pathogenesis of SLE.

A unique finding of this study is the binding of E_2-BSA-DNA conjugate to naturally occurring anti-DNA antibodies derived form the sera of SLE patients.

The results indicated the recognition of altered conformation of the modified polymer. The E_2-BSA-DNA conjugate was an effective inhibitor in DNA-anti-DNA system. So far, the possible explanations put forth for the production of anti-DNA autoantibodies have focussed mainly on the modification of DNA either as a result of environmental factors, hydroxyl/free radicals, radiations or some other factors including genetic and viral (Steinberg, 1992; Ara and Ali, 1992; Theofilopoulous, 1995). It is quite possible that in diseased state there may be altered conformation of estradiol-DNA conjugate rendering the hormone-receptor-DNA complex as 'alien' to the immune system, which might account for the predominance of SLE in females and also for the production of antibodies that cross-react with DNA. Compared to native DNA, SLE autoantibodies exhibited enhanced binding to ß-estradiol in ELISA experiments performed by using both colorigenic and fluorogenic substrates. It has been reported that the fluorogenic substrate increases the sensitivity of ELISA

(Ali and Ali, 1983). This provides further credence to the emerging hypothesis that native DNA is not the antigen responsible for the production of autoantibodies in SLE. This finding lends further support to the view that female sex hormone, might in one way or the other, be responsible for the production of lupus autoantibodies as the disease is more prevalent in women. It is possible that some sex hormonal disturbances that could occur as a result of contraceptive therapy, irregular menstrual cycle, child birth etc. might be contributing towards the production of autoantibodies. What appears from the studies undertaken so far and from the present work is that SLE is not a consequence of a single factor. The disease seems to be the result of various factors acting together or it is also possible that a single factor, might be leading to a chain of reactions involving other factors. The finding of Waters et al.(1992) wherein they have reported increased polyamine levels as a result of estradiol treatment to Atlantic salmon (Salmo salar), a fish, provides a direct relation between increased estradiol concentration and elevated polyamine levels in the body. If such a condition could also occur in humans than one might be able to explain and correlate the female predominance of SLE and the Z-DNA binding of anti-DNA antibodies from lupus patients, as it is well documented that high polyamine levels induce B→Z transition.

Based on this study the following points of conclusion could be drawn :

1. Induction of Z-conformation in native calf thymus DNA depends on its microenvironment. Z-DNA can be formed either by bromination in high sodium chloride concentration or as a result of polyamine interaction with DNA under physiological conditions of saline. Z-conformation in native calf thymus DNA is maintained even when the high salt brominated polymer is dialyzed in a buffer containing 0.15 M NaCl.
2. The binding of SLE anti-DNA antibodies to Z-conformer suggest that Z-DNA may be a potential putative autoantigen for the induction of antibodies cross-reactive with native DNA.
3. The modified forms, viz., DNA-spermine and E_2-BSA-DNA conjugate are thermodynamically more stable than their respective controls, i.e native DNA and DNA fragment(~200bp).
4. The binding of naturally occurring autoantibodies to E_2-BSA-DNA as well as the interaction of these antibodies with ß-estradiol clearly points towards the role of female sex-hormone in the pathogenesis of SLE.
5. It is suggested that female sex-hormone, either alone or in conjugation with DNA, might be challenging the immune system leading to autoantibody production.

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